In vitro toxicogenomics : unravelling the mechanisms underlying drug-induced hepatotoxicity

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In Vitro Toxicogenomics

Unravelling the mechanisms underlying drug-induced hepatotoxicity

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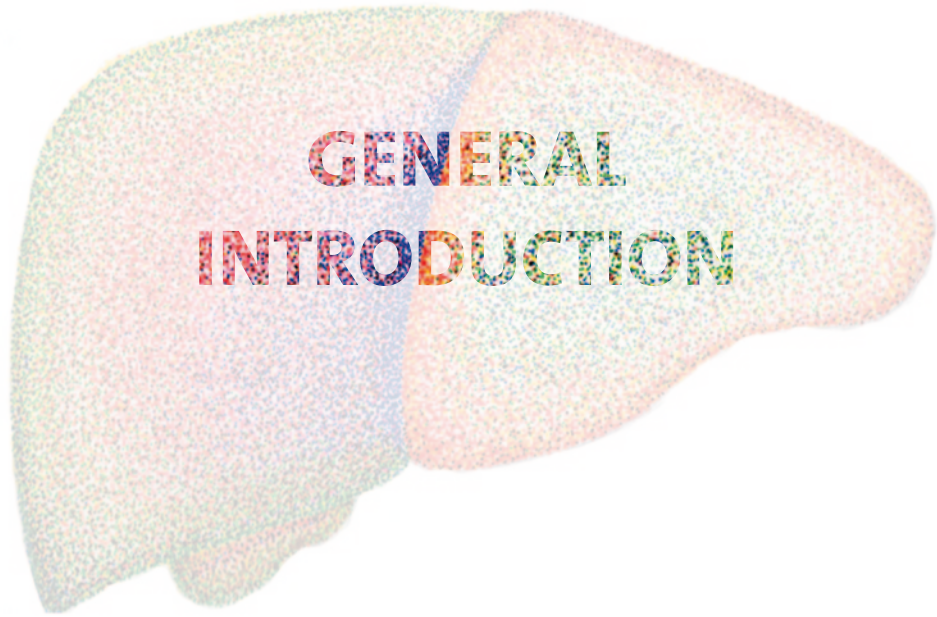
Contents

Chapter 1



Drug-induced hepatotoxicity

Drug development is a time-, and money-consuming business; it may take several years and up to US $ 800 million before a single new drug is approved and released onto the market. [1] Before a new compound can enter the market, it is extensively tested in preclinical and clinical trials. Preclinical testing mostly consists of long-term animal treatments. When no adverse effects are seen in laboratory animals, the compounds advance towards clinical human trials, which proceed through three phases. In the first phase, safe dosages, kinetics and toxicity of the compound are assessed in a small number of volunteers. The second phase is conducted in a larger number of people, who are diagnosed with the targeted disease. In this phase, the safety and efficacy is tested. In the last phase, the efficacy is further tested and patients are monitored for side-effects. Overall, on average 20% of all new drugs tested in clinical trials are successful after phase III and are approved for marketing. [2]

In a review of causes for project termination during clinical trials, toxicity is the second most indicated reason after lack of efficacy. [3] In approximately one third of these cases, hepatotoxicity is indicated as cause for project termination, rating drug-induced hepatotoxicity one of the major reasons for drug withdrawal. [4-6] The liver metabolizes and excretes drugs, making it vulnerable for adverse drug effects. Accumulation of drugs or the formation of toxic drug metabolites by hepatic enzymes can induce liver injury. Only a low percentage of drug-induced liver injuries is predictable and dose-dependent. Most drug-induced liver injuries occur infrequently and are considered to be idiosyncratic. Idiosyncratic drug-induced liver injury occurs at an incidence level of 1 in every 1000 to 1 in every 100,000 patients receiving therapeutic doses, which is another reason why these adverse drug reactions are often missed during clinical trials. [5] Different classes of drug-induced liver injury are considered, including necrosis, steatosis, cholestasis, inflammation or a combination of these.

Necrosis has been defined as an uncontrolled form of cell death in comparison to programmed cell death, named apoptosis. However, increasing evidence indicates that programmed cell death can occur with non-apoptotic features and that necrosis may be regulated by signal transduction pathways and catabolic mechanisms. [7] Necrosis is usually involved in acute drug-induced hepatotoxicity and is characterized by cellular and organelles swelling and rupture of the plasma membrane. [8]

Steatosis is characterized by the intracellular formation of lipid droplets in the cytoplasm, which is believed to be the result of inhibition of ß-oxidation of fatty acids or inhibited secretion of fatty acids by very-low-density-lipoprotein particles. [9]

Cholestasis is one of the most severe manifestations of drug-induced liver injury because of a high mortality rate and may account for 16% or up to 50% of all cases of drug-induced hepatotoxicity. [10] Cholestasis is characterized by the intracellular accumulation of substances normally excreted via the bile, such as bile acids, cholesterol, bilirubin, and drug metabolites. This intracellular accumulation may be the result of drug-induced inhibition of transport proteins. One of the most important transport proteins for bile acids is the bile salt export pump (BSEP). Several cholestasis inducing drugs were shown to inhibit the function of the

BSEP. [11] Recently, Vinken et al. published an adverse outcome pathway for drug-induced cholestasis resulting from BSEP inhibition.[12]

Increasing evidence indicates that the immune system plays an important role in drug-induced liver injury. Activation of immune cells and recruitment to the liver can cause inflammation; however, the mechanisms involved are largely unknown. It is hypothesized that drug-induced cell necrosis causes release of cell debris, which can activate and recruit immune cells. Recruited cells may attack stressed liver cells and thereby induce additional liver damage. [13]

In vitro cell lines

Before a candidate-drug enters clinical trials, drugs are extensively tested in animal toxicity studies, ranging from acute toxicity tests to long-term toxicity testing. However, these studies are time-consuming and the use of laboratory animals for toxicity testing is subject to an ongoing ethical debate. Furthermore, although Olson et al. [14] showed a true positive concordance rate of up to 71% for human toxicity with animal models, the overall sensitivity and specificity of (animal) tests for human toxicity should be improved since liver toxicants still progress into clinical trials. [15] Therefore, a major effort is put in finding more reliable, and preferably, non-animal-based *in vitro* tests, which are predictive for human *in vivo* toxicity and can be used for the investigation of toxicological mechanisms. Several *in vitro* liver models summarized in Table 1, will be discussed next.

Liver slices

Liver slices are believed to represent a valuable *in vitro* liver model since they contain all cells present in the liver and the slices retain their three dimensional structure. Furthermore, liver slices express phase I and phase II enzymes while also other liver-specific functions are preserved, such as albumin and glucose production. [16] Boess et al. report that the mRNA expression profiles of rat liver slices are more comparable to rat liver *in vivo* than two rat cell lines. [17] However, the preparation and culture conditions of liver slices are laborious and effort is put in optimizing this, resulting in many different protocols which thus involves the risk that this results in large inter-laboratory differences. [18] Furthermore, even with optimized protocols, loss of cytochrome P450 is already high after 24 hours and cell death occurs after culturing for 48-72 hours, making liver slices unusable for long exposures. [19-20]

Primary human hepatocytes

When investigating drug-induced hepatotoxicity in humans *in vitro*, primary human hepatocytes (PHH) are considered to represent the best *in vitro* cell system. [21-22] When cultured in a sandwich-culture, PHH show largely the same behavior as hepatocytes *in vivo*, including the metabolization of and response to drugs. [23-24] However, their use in toxicological research is bound to limitations. Fresh human liver samples are not always available and culturing procedures are laborious. Furthermore, the use of PHH is costly and comparing multiple experiments is difficult due to inter-individual differences between liver donors. [25]

Stem cells

When using primary hepatocytes, one depends on donors and even though cells can be frozen and used later, the number of cells is limited. Another option for generating hepatocytes may be the use of stem cells. In this relatively new field, two models are mostly investigated, namely embryonic stem cells and induced pluripotent stem cells. Metabolic active hepatocyte-like cells can be produced using both models. [26-27] However, different differentiation protocols result in major differences between the hepatocyte-like cells and the final culture will contain stem cells next to differentiated liver cells. Furthermore, the use of embryonic stem cells is subject to an ethical debate. Moreover, the metabolic functionality of the hepatocyte-like cells will decrease over time, comparable to cultures of primary hepatocytes. [28] Optimization of differentiation protocols may further improve the functionality of hepatocyte-like cells produced from stem cells, which may lead to the use of these cells in high-throughput toxicity screenings.

Immortalized cell lines

The human hepatocellular carcinoma cell line HepG2 and the commercially available HepaRG cell model are thought to present acceptable alternatives, since both represent a human cell model and are easy and cheap to culture. Research has shown that HepG2 cells show less metabolization of compounds compared to PHH, in association with low levels of specific enzymes involved in phase I and II metabolism. [29-30] However, several studies demonstrated that HepG2 cells were able to metabolize drugs leading to toxic effects [31-34] and this cell line is still used in many toxicological studies. [35-46] Furthermore, HepG2 cells and PHH showed similarly modulated gene sets after treatment with a toxic compound, suggesting similar drug-induced effects between these two cell types. [47] HepaRG cells show higher baseline levels of phase I and phase II enzymes in comparison to HepG2 cells; however, when exposed to toxicants, the responses of both cell lines are comparable. [48-49]

Primary hepatocytes from rodents

Since animal-based toxicity screenings are most often performed with rodents, it is suggested that primary hepatocytes from rodents present a relevant alternative *in vitro* liver model. Primary hepatocytes from rodents cultured in a collagen sandwich configuration have the same advantages as PHH, including good metabolic competence. Furthermore, primary hepatocytes from rodents are easily available, have a low interindividual variation and knockout models can be used to investigate specific genotypes and mechanisms. Mathijs et al. showed that primary mouse hepatocytes (PMH) are metabolic more stable than primary rat hepatocytes and maintain their metabolic competence for up to 90 hours, making PMH more useful as an *in vitro* liver model. [50] Toxicity screening using PMH facilitates the screening of multiple compounds using one single mouse, resulting in reduced numbers of laboratory animals needed for these screenings. However, extrapolating mouse *in vitro* results to human *in vivo* mechanisms might be complicated and should be done with care.

Table 1. *Advantages and disadvantages of in vitro liver models.*

Model	Advantages	Disadvantages
Liver slices	*Three dimensional structure* *All liver cell types*	*Preparation and culturing laborious* *Only short term exposures*
Primary human hepatocytes	*Maintain hepatocyte-specific functions* *Comparable to in vivo liver*	*Interindividual differences* *Low availability*
Stem cells	*Unlimited production of hepatocytes* *Expression of phase I and phase II enzymes*	*Many protocols results in differences in cells* *Cultures contain mix of stem cells and hepatocytes* *Embryonic stem cells: ethical debate*
Immortalized cell lines	*Easy and cheap to culture* *Human cell model*	*Lower expression of phase I and phase II enzymes*
Primary hepatocytes from rodents	*Easy available and low variability* *Expression of phase I and phase II enzymes*	*Hepatocytes from rodents*

Toxicogenomics

Traditionally, drug-induced liver injury in preclinical studies and clinical trials is assessed using several parameters, e.g. clinical chemistry and pathology. [51] However, identification of hepatotoxicity relies on the integration of all study data and a correct interpretation. [52] Traditional biomarkers for *in vivo* liver injury are alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyltransferase, bilirubin, ammonia and a prolonged prothrombin time. Although these biomarkers can give valuable information about liver damage, their specificity and sensitivity are far from ideal and differentiation between different liver injury phenotypes is difficult. [53-54] Furthermore, these traditional parameters mostly reflect late responses and endpoints when irreversible cell damage has already occurred. Moreover, traditional biomarkers may not be sensitive enough to detect cell damage *in vitro* due to the low number of cells used in *in vitro* models. Drug-induced toxicity is the result of altered biological processes by a compound or its metabolites, resulting in adverse effects. Therefore, the investigation of drug-induced alteration of biological processes will further improve the identification of hepatotoxic compounds. For this, toxicogenomics which refers to the combination of toxicology with genomics, involving transcriptomics, proteomics, and metabonomics, is considered promising. [55]

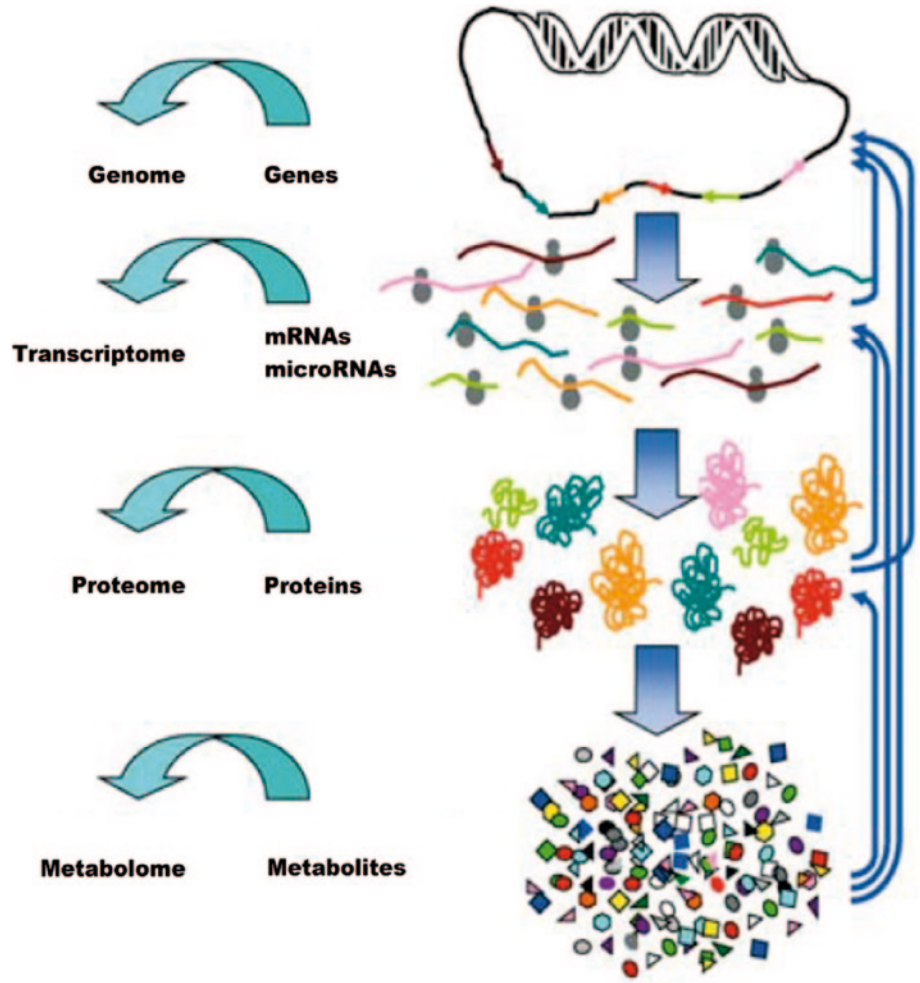


Figure 1. *In Toxicogenomics, toxicology is combined with genomics, transcriptomics, proteomics and metabolomics. Since all these molecules interact with each other, it is believed that integrated data analysis of all omics platforms is needed to fully elucidate the mechanisms underlying drug-induced liver injury. Figure is adapted from Goodacre.* [56]

Transcriptomics

Transcriptomics is defined as the profiling of DNA transcripts or RNA molecules. Profiling of messenger RNA (mRNA) levels also known as gene expression profiling, is most common. Gene expression profiling using microarrays is still the most used technological platform in toxicogenomic research. [57] It is a robust technique [58] and has frequently been used to investigate drug-induced liver toxicity. [59-61] Transcriptomic research is still expanding and also includes

profiling of other RNA molecules, such as microRNAs. MicroRNAs can regulate mRNA levels and mRNA translation to proteins and therefore may play an important role in cellular mechanisms, including drug-induced toxicity. [62] The recent introduction and advances of next generation sequencing of RNA molecules has the potential to further improve the transcriptomic field, where sequencing can lead to the discovery of new transcripts involved in drug-induced toxicity. [63]

Proteomics

Transcriptomic profiles can give an indication of protein expression; however, true protein levels as well as post-translational modifications need to be measured by means of proteomic technologies. Proteomics is applied to toxicology and has been proposed as technique to give valuable information about the mode of action of compounds and to discover biomarkers of drug-induced hepatotoxicity. [64-65] In order to find new biomarkers using untargeted analysis of drug-induced changes on protein expression, cellular proteins need to be separated, quantified and identified, which can be done using multiple techniques (e.g. Liquid Chromatography coupled to (Tandem) Mass Spectrometry or Difference Gel Electrophoresis). [66] Targeted proteomics can be used to validate potential biomarkers or investigate a specific subset of proteins. [67]

Metabonomics

Metabonomics is defined as 'the quantitative measurement of the time-related multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification' [68] and is applied to toxicology to investigate drug-induced metabolite changes. Metabonomics has to potential to be used in high-throughput toxicity screening, identification of biomarkers of toxicity and further elucidation of mechanisms of action. [69] Mass spectrometry and Nuclear Magnetic Resonance spectroscopy are the most applied techniques in targeted and untargeted metabonomics and each technique has its own advantages and disadvantages. [70]

Integrative analyses

Although every omic technology individually provides valuable information about drug-induced changes, it is believed that their true value lies within an integrative analyses of multiple omics, ultimately evolving into a complete systems biology approach. [51, 71] Several publications showed that integrative analyses of multiple omics data of *in vivo* drug-induced liver injury in rodents resulted in a better understanding of the underlying mechanisms. [72-74] Analysis of transcriptomics and metabonomics data in HepG2 experiments confirmed the added value of integration *in vitro*. [75] Overall, integrated data analysis has the potential to fully elucidate mechanisms underlying drug-induced hepatotoxicity and improve the preclinical screening of new drugs.

Model compounds

In order to further elucidate mechanisms underlying specific hepatotoxic phenotypes, the use of prototypical drugs that induce these phenotypes *in vivo* is essential. Model compounds known to induce necrosis, steatosis or cholestasis in humans and negative controls were selected based on information from the National Toxicology Program (http://ntp.niehs.nih.gov/) and literature. [76-77] To introduce these prototypical drugs, we will describe a number of hepatotoxicants.

Necrosis-inducing compounds

Acetaminophen

Acetaminophen (APAP) is an over-the-counter available non-steroidal anti-inflammatory drug (NSAID) and analgesic which is not toxic at a therapeutic dose. However, at high doses APAP can induce sever liver damage and APAP overdose is one of the most common causes of acute liver failure in the US and Europe. [78-79] The mechanism underlying APAP-induced necrosis is thought to be the result of glutathione depletion by reactive metabolites formed in the metabolism of APAP by CYP enzymes. Depleted glutathione levels lead to increased oxidative stress, which results in ATP loss and eventually necrosis. [80]

Diclofenac

Diclofenac (DIC) is an analgesic and NSAID frequently prescribed for the treatment of rheumatoid disorders and known to induce hepatic necrosis. Although mechanisms underlying DIC-induced liver injury are not fully understood, reactive metabolites, oxidative stress and mitochondrial failure are thought to be involved. [81]

Isoniazid

Isoniazid (ISO) is used in the prevention and treatment of tuberculosis and is metabolized in the liver. The metabolite hydrazine is believed to be responsible for the ISO-induced hepatic necrosis. [82]

Paraquat

Paraquat (PAQ) is a widely used herbicide and high dose and prolonged exposure can induce hepatic necrosis is humans and rodents. [83-84] Liver injury is believed to be the result of oxidative stress by free radical formation and glutathione depletion. [85]

Steatosis-inducing compounds

Amiodarone

Amiodarone (AM) is an antiarrhythmic drug effective in several cardiac tachyarrhythmias's and thus regularly prescribed. AM accumulates in fat tissue and due to the high fat content of the liver, this is one of the prominent organs where AM accumulates and induces steatosis which may evolve in liver cirrhosis. [86]

Tetracycline

Tetracycline (TET) is an antibacterial drug which by itself or through one of its metabolites inhibits mitochondrial fatty acid oxidation resulting in microvesicular steatosis. [87]

Valproic Acid

Valproic acid (VPA) is one of the most widely prescribed antiepileptic drugs. The mechanisms underlying VPA-induced microvesicular steatosis are not completely understood, however, impairment of mitochondrial function and fatty acid metabolism have been observed *in vivo* and *in vitro*. [88]

Cholestasis-inducing compounds

Cyclosporin A

Cyclosporin A (CsA) is a widely used immunosuppressant primarily used to prevent rejection in organ transplantation. CsA-induced cholestasis was observed in transplant patients and in animal experiments. [89-90] CsA was shown to inhibit several ATP-dependent export carriers, including the BSEP. [91-92] As previously described, inhibition of transport proteins can lead to an intracellular accumulation of bile-acids resulting in hepatocellular damage and cholestasis. [93]

Chlorpromazine

Chlorpromazine (CPZ) is an antipsychotic drug and one of the most prescribed drugs in the treatment of schizophrenia worldwide. [94] CPZ-induced hepatotoxicity has been associated with hypersensitivity resulting in cholestatic hepatitis, although CPZ-induced cholestasis also seems to involve a toxic component. [95]

Ethinyl Estradiol

Ethinyl estradiol (EE) is an orally bioactive estrogen derivative used in oral contraceptive pills. Estrogens are known to induce intrahepatic cholestasis through impairment of bile salt dependent and independent bile flow via multiple mechanisms. [96]

Hypothesis and outline of thesis

Drug discovery and development are time-consuming and expensive processes, which involve the design and production of the drug and its testing for efficacy and safety in pre-clinical research and in subsequent clinical trials. Drug-induced hepatotoxicity appears one of the most indicated reasons for drug withdrawal during clinical trials or even after admittance onto the market. Discovery of hepatotoxic potential of new drugs at an early time point may therefore save considerable time and resources. Animal toxicity testing based upon traditional endpoints remains to be the standard in pre-clinical toxicity testing, in despite of large number of drugs missed as hepatotoxic. Relatively new technologies such as transcriptomics, proteomics and metabolomics providing a large amount of new endpoints are expected to generate better predictors for human toxicity and are being used more and more in preclinical safety research. This PhD project is based on the hypothesis that the combination of transcriptomics, proteomics and metabonomics in an integrative *in vitro* toxicogenomics approach will improve the knowledge of mechanisms underlying drug-induced hepatotoxicity. This may lead to better *in vitro* hepatotoxicity prediction and classification of hepatotoxicants.

The HepG2 cell model is frequently used in hepatotoxicity studies, is easy to culture and has previously been used to predict the genotoxic properties of compounds based on the omics approach. Therefore, transcriptomic profiling of HepG2 cells exposed to hepatotoxic and non-hepatotoxic compounds was used to predict hepatotoxic potential of compounds, which is described in **Chapter 2**.

Where the metabolic capacity of primary mouse hepatocytes is better than the metabolic capacity of HepG2 cells, **Chapter 3** compares gene expression analyses of three cholestatic compounds in HepG2 cells and primary mouse hepatocytes to further unravel the mechanisms underlying drug-induced cholestasis in two *in vitro* liver cell models.

In **Chapter 4** transcriptomic and metabonomic analyses of HepG2 cells exposed to the cholestatic compound cyclosporin A were integrated to investigate the added value of multi-omics analysis.

In **Chapter 5**, we exposed primary mouse hepatocytes to cyclosporin A and used an integrated transcriptomics, proteomics and metabonomics approach to further unravel the mechanism underlying cyclosporin A induced hepatotoxicity.

The three hepatotoxic phenotypes necrosis, steatosis and cholestasis were further investigated using integrated multi-omics analyses in order to elucidate different and comparable mechanisms induced by drugs. Two *in vitro* cell models, HepG2 cells and primary mouse hepatocytes, were exposed to three prototypic drugs and transcriptomic and proteomic results were integrated as described in **Chapter 6**.

Finally, the work presented in the previous chapters is summarized in **Chapter 7** and the results are discussed in view of previous work and future research directions are suggested.

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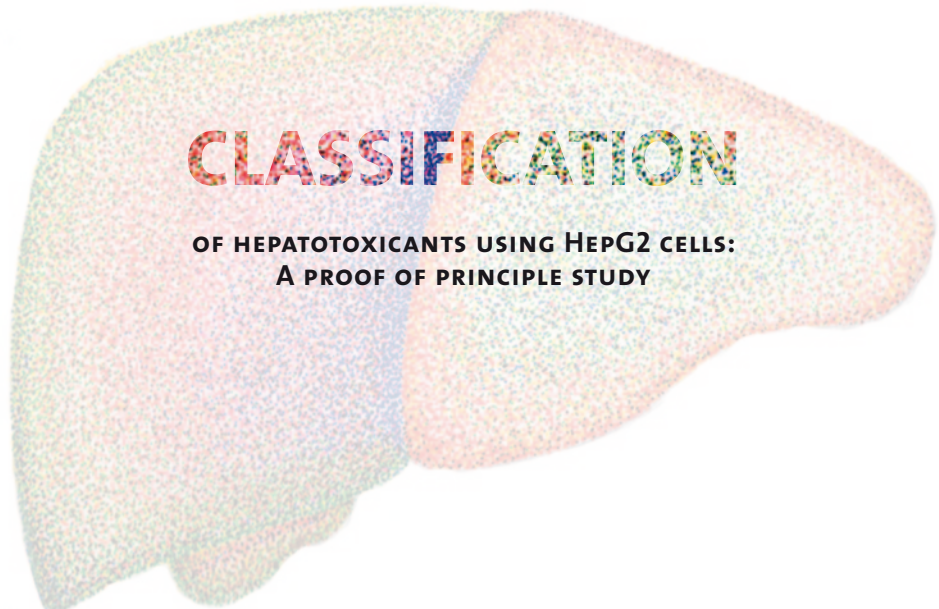
Chapter 2



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Abstract

With the number of new drug candidates increasing every year, there is a need for high-throughput human toxicity screenings. As the liver is the most important organ in drug metabolism and thus capable of generating relatively high levels of toxic metabolites, it is important to find a reliable strategy to screen for drug-induced hepatotoxicity. Microarray-based transcriptomics is a well-established technique in toxicogenomics research and is an ideal approach to screen for drug-induced injury at an early stage. The aim of this study was to prove the principle of classifying known hepatotoxicants and non-hepatotoxicants using their distinctive gene expression profiles *in vitro* in HepG2 cells. Furthermore, we undertook to sub-classify the hepatotoxic compounds by investigating the subclass of cholestatic compounds. Prediction Analysis for Microarrays was used for classification of hepatotoxicants and non-hepatotoxicants, which resulted in an accuracy of 92% on the training set and 91% on the validation set, using 36 genes. A second model was set up with the goal of finding classifiers for cholestasis, resulting in 12 genes that appeared capable of correctly classifying 8 of the 9 cholestatic compounds, resulting in an accuracy of 93%. We were able to prove the principle that transcriptomic analyses of HepG2 cells can indeed be used to classify chemical entities for hepatotoxicity. Genes selected for classification of hepatotoxicity and cholestasis indicate that endoplasmic reticulum stress and the unfolded protein response may be important cellular effects of drug-induced liver injury. However, the number of compounds in both the training set and the validation set should be increased to improve the reliability of the prediction.

Introduction

The number of new drug candidates increases every year and all these candidates need to be tested for safety. Testing that many candidates is time- and money-consuming and involves the use of high numbers of laboratory animals. Furthermore, these pre-clinical animal tests are no guarantee that the apparent non-toxic candidates will also be non-toxic in humans, as is demonstrated by the fact that many candidates are withdrawn during clinical trials or even later upon market introduction.

One of the major reasons for drug withdrawal is drug-induced hepatotoxicity. [1-2] The liver is the most important organ involved in drug metabolism and drugs may accumulate in the liver or toxic drug metabolites may be formed by enzymes in the liver. So, a reliable *in vitro* test for drug-induced hepatotoxicity would be a cheaper and faster alternative and could be used to effectively reduce the numbers of drug candidates advancing to animal tests and clinical trials.

Researchers have been looking for alternatives for animal-based liver toxicity tests for years, evaluating several *in vitro* models (e.g. primary hepatocytes, immortalized cell lines, hepatocellular carcinoma cell lines) in the process. The gold standard for *in vitro* toxicity testing is thought to be primary human hepatocytes as these would represent the human *in vivo* situation best. But primary human hepatocytes are difficult to obtain and thus expensive. Furthermore, inter-individual differences are high and culturing conditions are laborious. So, screening of large numbers of chemicals would be difficult using primary human hepatocytes. HepG2 cells represent a human hepatocellular carcinoma cell line, are cheap and easy to culture. Although it is known that HepG2 cells have low levels of specific enzymes involved in phase I and II metabolism [3-4], this cell line is still used in many toxicology studies. [5-8] Schoonen et al. showed that HepG2 cells can be used to sensitively screen for cytotoxicity by measuring the Reactive Oxygen Species (ROS) formation, glutathione depletion and membrane integrity. [6] ROS formation and glutathione depletion are two important mechanisms of drug-induced hepatotoxicity and have consequent effects on cellular organelles. [9] Furthermore, Liguori et al. reported similarly modulated gene sets in primary human hepatocytes and HepG2 cells after treatment with the toxic compound trovafloxacin, suggesting similarities in drug-induced effects between these two cell types. [10]

Although many studies use functional endpoints to screen for drug-induced cell damage, transcriptomics is the ideal tool to screen for drug-induced injury at an early stage, because gene expression analysis can give valuable information about the multiple mechanisms underlying the drug-induced toxicity. [11] Transcriptomics is a well-established technique in toxicogenomics research and has been used to identify gene expression patterns of hepatotoxicants after short-term rodent experiments. [12-14] Zidek et al. reported an accurate discrimination between acute hepatotoxic compounds and non-hepatotoxicants based on the gene expression profiles in rat livers 24 hours postadministration. [15] Furthermore, Steiner et al. confirmed that *in vivo* discrimination of different classes of toxicants is feasible using gene expression patterns in rat livers. [16] Previous work from our department showed that also *in vitro* transcriptomic analysis of HepG2 cells combined with results from the Ames mutagenicity test is capable of successfully predicting the genotoxic hazard of compounds *in vivo*. [17] Furthermore, Sawada et al. developed

an *in vitro* screening test for phospholipidosis using gene expression analyses of HepG2 cells exposed to toxic compounds. [18] All in all, we considered it worthwhile to investigate the HepG2 liver model for its relevance for assessing hepatotoxicants based on the toxicogenomics approach.

Therefore, the aim of this study was to prove the principle of classifying known hepatotoxicants and non-hepatotoxicants *in vitro* using their distinctive gene expression profile in HepG2 cells after 24 hours of incubation. Furthermore, we aim to evaluate the classification of the important subclass cholestasis. Prediction Analysis for Microarrays (PAM) was used for classification of hepatotoxicants and non-hepatotoxicants. PAM uses the nearest shrunken centroid method and was used by Tibshirani et al. to diagnose multiple cancer types. [19]

Materials and methods

Chemicals

Modified Eagle's medium (MEM) plus glutamax, sodium pyruvate, fetal calf serum (FCS), non-essential amino acids, penicillin/streptomycin, Hanks' calcium- and magnesium-free buffer were obtained from Invitrogen (Breda, The Netherlands), dimethylsulfoxide (DMSO), Trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Acetaminophen, Amiodarone, Chlorpromazine, Cyclosporin A, Diclofenac, D-Mannitol, Ethinyl Estradiol, Isoniazid, Lithium Carbonate, Paraquat, Tetracycline and Valproic Acid were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Adefovir was a gift from Leiden University.

Training set

HepG2 cells were cultured in MEM plus glutamax containing 10% v/v FCS, 1% v/v Sodium Pyruvate, 1% v/v non-essential amino acids, 2% v/v penicillin and streptomycin at 37°C in an atmosphere containing 5% CO_2.

Compound concentrations were derived from cytotoxicity tests based on the MTT method as described in an earlier study [20] and results for the 24 hours incubation can be found in Table 1. HepG2 cells were seeded in 6 well plates and were treated for 24 hours with the IC_{20} concentrations measured after 24 hours. Compounds were dissolved in DMSO or PBS and added to the medium with a final concentration of 0.5% v/v DMSO or PBS. Cells incubated in the presence of 0.5% v/v DMSO or PBS served as solvent controls. These experiments were performed with three replicates which were from independent cultures, with HepG2 passage numbers between 8 and 14. HepG2 cells were treated with 10 hepatotoxic compounds (Acetaminophen, Amiodarone, Chlorpromazine, Cyclosporin A, Diclofenac, Ethinyl Estradiol, Isoniazid, Paraquat, Tetracycline, Valproic Acid) and 3 non-hepatotoxic compounds (Adefovir, D-Mannitol, Lithium Carbonate).

Total RNA isolation, target preparation and microarray hybridization on the Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays and scanning on an Affymetrix GeneArray scanner, were performed according to standard procedures. Quality control was performed using the ArrayAnalysis.org web service [21] including Robust Multi-array Average (RMA) normalization and analyses of the default parameters indicated that all microarrays were

of high quality. The arrays contained a total of 1,354,896 probes, which after custom CDF annotation and normalization resulted in 18,926 genes.

Validation set

CEL-files of HepG2 cells exposed to 6 hepatotoxic compounds (17-beta Estradiol, Azathioprine, Cisplatin, Cyclophosphamide, Cyclosporin A and Phenobarbital) and 2 non-hepatotoxic compounds (D-Mannitol and Mitomycin C) were obtained from the study of Magkoufopoulou et al.[17] From in-house studies, two additional data sets on hepatotoxic compounds (Nifedipine and Tolbutamide) and one non-hepatotoxic compound (Clonidine) were added. For this set of compounds HepG2 cells were exposed for 24 hours to the IC_{20} concentrations measured after 72 hours (see Table 2).

Classification

All pre-processing analyses were performed using R version 2.15.3 for Windows (64-bit). [22] Several packages were used for this analysis (Affy v1.36.1, SimpleAffy v2.34.0, Affycomp v1.34.0) and were all acquired from Bioconductor, version 2.11. [23] A total of 105 Affymetrix CEL-files were obtained and were reannotated to EntrezGene IDs using Brainarray's custom CDF version 15.1.0, as described by Dai et al. [24] and normalized using the RMA method combined with MAS5-PMA-calling. [25] Only present and marginal probes were selected for further analysis and their intensities were transformed into LogRatios compared to matching controls.

Preselection of features

To rule out compound-specific effects contributing to gene expression changes, a leave-1-compound-out t-test ($p<0.01$) was performed as described previously. [17] Therefore, the set was divided in 2 groups: hepatotoxic and non-hepatotoxic compounds. To validate the results of the leave-1-compound-out t-test, we compared the results with an additional approach using a moderated t-test. The amount of significant features was lower in the moderated t-test, however, more than 80% of the features were overlapping with the list of the leave-1-compound-out t-test, which indicates the robustness of this approach. Genes that were significant in all of the leave-1-compound-out t-tests were selected for classification.

Class prediction

The preselected set of features was loaded into PAM (Prediction Analysis for Microarrays, version 2.21) for class prediction. [19] The data were trained and the model was chosen based on the best threshold matching our criteria of A) a low misclassification error and B) a strong reduction in amount of features. Hepatotoxic versus non-hepatotoxic classification threshold was 2.273, resulting in a reduction of features from 740 to 36. Cholestasis classification threshold was 3.126, resulting in a reduction of features from 853 to 12. The validation set was predicted using the features in this model. Final compound-class was assigned by at least 2 out of 3 biological replicate experiments. The distribution of the performance estimates could not be assessed, since an external validation set from a different experiment was used and no multiple splits of

the data into training and validation sets were performed.

Biological interpretation

Pearson/Ward hierarchical clusters were generated within GenePattern [26] based on the log2 fold changes (Additional file 1-2). For the biological interpretation of the selected genes for the classification of hepatotoxic compounds, a network was created using the MiMI-plugin [27] in Cytoscape (version 2.8.3). [28] The selected features were uploaded into the MiMI-plugin and a network was created based on known biological interactions including nearest neighbors shared by more than one gene. For visualization of the different effects of hepatotoxic and non-hepatotoxic compounds, the ExprEssence-plugin [29] was used to add gene expression data to the network based on the fold changes between exposed cells and the solvent controls.

For further biological interpretation of the features selected for the classification of hepatotoxicity and for the features selected for the classification of cholestasis, Gene ontology terms [30] associated with the selected genes were explored.

Results

PAM was applied to select gene signatures for prediction of drug-induced hepatotoxicity. The accuracy, sensitivity, false negative rate, specificity, false positive rate, positive predictive value, and negative predictive value are presented in Table 3. This prediction model has an accuracy of 92% for the training set and 91% for the validation set for the prediction of hepatotoxicity. A specificity of 100% was found because there were no false positive predictions. Sensitivity is also high, only one false negative in the training set, isoniazid, and one in the validation set, cisplatin, which results in a sensitivity of 90% and 88% for the training set and validation set, respectively. A total of 36 genes were selected and these are summarized in Table 4.

Hierarchical cluster analyses of the genes in the training set and validation set show different gene expression patterns for hepatotoxic and non-hepatotoxic compounds (Figure 1). In the training set, the hepatotoxic compounds paraquat and isoniazid cluster together with the non-hepatotoxic compounds. This may be explained by the fact that the overall LogRatios of these two hepatotoxic compounds for the selected classifiers are low compared to the other hepatotoxic compounds.

For biological interpretation a network was built by means of Cytoscape using the MiMI plugin. All classifiers were uploaded and were supplemented with their nearest neighbors shared by more than one gene. The created network comprises 21 of the 36 genes, supplemented with 33 neighbors (Figure 2). The other 15 genes did not show interactions with the 21 genes and 33 added neighbors and did therefore not appear in this network. For the visualization of the different effects of hepatotoxic and non-hepatotoxic compounds on the selected features, two representative compounds were selected based on their high fold changes after treatment. For the non-hepatotoxic compounds, adefovir was chosen, and tetracycline was used as a representative for the hepatotoxic compounds, visualized on the left and right side of the colored nodes, respectively. There is an opposite effect between these two compounds on most of the genes in this network. The genes up-regulated after treatment with adefovir

and down-regulated after tetracycline treatment are mostly involved in cell cycle processes. Genes up-regulated after treatment with tetracycline and down-regulated after adefovir treatment were involved in several processes, including cell cycle processes. Furthermore, genes up-regulated after treatment with the hepatotoxic compound tetracycline were involved in the endoplasmic reticulum (ER) overload response, amino acid metabolism and transport.

For further biological interpretation, the Gene Ontology (GO) terms associated with the selected features were analyzed and the most relevant terms are summarized in Table 5. For four of the features selected for the classification of hepatotoxic compounds versus non-hepatotoxic compounds, INHBE, NEAT1, LOC100505650 and USP6NL-IT1, no GO terms were found. The other 32 features were associated with GO terms involved in cell cycle processes, cellular and ER stress, protein processing and lipid metabolism.

For exploring the feasibility of classifying cholestasis, a new model was trained and validated with PAM without the steatotic and necrotic compounds. The accuracy, sensitivity, false negative rate, specificity, false positive rate, positive predictive value, and negative predictive value are presented in Table 6. An accuracy of 100% was found for the training set and 78% for the validation set for this prediction model containing 16 genes. No false positives were identified, resulting in a specificity of 100% in both the training and validation set. Sensitivity was 100% in the training set and 67% in the validation set, due to 2 false negative compounds, namely 17-beta estradiol and phenobarbital. However, these results were based on a training set containing only three cholestatic and three non-hepatotoxic compounds. To reduce the risk of overtraining the model and focusing on compound-specific changes, all cholestatic and non-hepatotoxic compounds from the training set and validation set were combined. Leave-one-out t-tests and PAM were performed to see if cholestatic specific genes could be found which could be used to classify cholestatic and non-hepatotoxic compounds (results are summarized in Table 7). 12 genes were selected (Table 8) which were able to correctly predict the cholestatic properties of 8 of the 9 cholestatic compounds in the combined set; only 17-beta estradiol was incorrectly predicted as non-hepatotoxic. All non-hepatotoxic compounds were correctly predicted.

Most of the cholestatic compounds show the same gene expression pattern of the genes in the hierarchical cluster analysis (Figure 3). However, the cholestatic compound 17-beta estradiol shows low LogRatios for the selected genes and does not cluster together with the other cholestatic compounds. For the cholestatic compounds azathioprine and phenobarbital respectively one and two of the three replicates do not cluster with the replicates of the cholestatic compounds. The non-hepatotoxic compounds show an opposite gene pattern for the selected genes when compared to the cholestatic compounds or show low LogRatios indicative of a low response of these genes to the specific compounds.

GO terms associated with the 12 selected genes were explored for the biological interpretation (see Table 9). Five of the selected genes for classification of cholestasis were directly associated with GO terms involved in the unfolded protein response (UPR). No GO terms were found for the INHBE gene. GO terms associated with the other 6 genes were involved in cellular metabolism, response to drugs and cell cycle processes.

Discussion

In this study, we aimed to classify hepatotoxic and non-hepatotoxic compounds using transcriptomic profiles of exposed HepG2 cells. Compounds were selected based on information from databases from the National Toxicology Program (http://ntp.niehs.nih.gov/) and literature. [31] Finding the appropriate representative candidates for non-hepatotoxic compounds and the different hepatotoxic classes is not trivial. Some negative controls for hepatotoxicity are not toxic for the liver but may be toxic for other organs. This toxicity may include comparable modes of action and differential gene expression, which may interfere with the classification of hepatotoxicants from non-hepatotoxicants. Furthermore, D-mannitol is an inert compound, where Mitomycin C can induce chromosomal damage, which shows that there are also major differences between the selected non-toxicants. Building microarray based databases that consist of gene-expression data from hepatocytes exposed to well-known hepatotoxicants and non-hepatotoxicants could therefore further improve the prediction of newly developed compounds as has been done by some groups for rat livers. [32-34]

Predicting hepatotoxicity resulted in an accuracy of 92% in the training set, where only one hepatotoxic compound, isoniazid, was predicted as non-hepatotoxic. The metabolites of isoniazid formed by CYP3A4 are more toxic than the compound itself. CYP3A4 activity is low in HepG2 cells [35], which could possibly result in lower levels of metabolites and lower LogRatios of the selected genes after isoniazid treatment which could consequently be responsible for the wrong prediction. Amiodarone was correctly predicted as hepatotoxic, but LogRatios of the selected genes are low compared to the other hepatotoxic compounds, which also could be due to the low activity of CYP3A4 in HepG2 cells. [36]

In the validation set, an accuracy of 91% was found for the prediction of hepatotoxicity. The hepatotoxic compound Cisplatin was incorrectly predicted as non-hepatotoxic. In contrast to the training set where the IC_{20} after 24 hours was used as the incubation concentration, in the validation set HepG2 cells were treated for 24 hours with the IC_{20} established after 72 hours of exposure, which may be lower than the IC_{20} after 24 hours. As can be seen in the hierarchical cluster analyses, some compounds, including Cisplatin, show only minor changes of the genes used for the classification of hepatotoxicity. Preferably, a comparable concentration selection should be used, however, no such data set was available at this time.

For biological interpretation a network was created to assess the interactions between the 36 selected genes. 21 of the 36 genes could be added to the network including 33 neighbors, suggesting possible indirect interactions between these 21 genes. The down-regulation of genes involved in cell cycle processes after exposure to hepatotoxic compounds may indicate cell cycle arrest, which is an indication of induced toxicity. Furthermore, genes involved in ER stress were up-regulated after treatment with hepatotoxic compounds also indicative of a stress response to the hepatotoxic compounds. ER stress could result in an accumulation of unfolded proteins and subsequently to an unfolded protein response. General protein translation is down-regulated by the UPR, which results in lower expression of proteins with short half-lives. Many of these proteins are involved in the cell cycle and a reduction of these cell cycle proteins will result in cell cycle arrest. [37-38]

For 32 of the selected features of the first classification step associated GO terms were investigated which revealed mechanisms indicative for hepatotoxicity like cellular and ER stress, UPR and lipid metabolism. The GO terms associated with ER stress and the UPR signify the UPR as an important event in drug-induced hepatotoxicity. It is suggested that the UPR is also capable of modulating lipid metabolism [39], further emphasizing an important role for the UPR in drug-induced toxicity. For four features, INHBE, NEAT1, LOC100505650 and USP6NL-IT1, no GO terms were found and literature was searched to identify possible associated mechanisms. Bruning et al. showed that INHBE was up-regulated by drug-induced ER stress [40] and INHBE was also up-regulated in HepG2 cells exposed to hepatotoxicants. NEAT1 is in Mus Musculus associated with the GO term paraspeckles. Fox et al. suggest that paraspeckles have a role in the regulation of translation [41], but the exact mechanisms are not clear. Nissim et al. showed up-regulation of LOC100505650 in hepatitis B virus associated acute liver failure [42], however this gene was down-regulated after treatment with hepatotoxicants. The function of USP6NL-IT1 is not yet understood. Further research should investigate the function of this gene and the possible relationship between up-regulation of this gene and hepatotoxicity.

The used validation set mostly consisted of cholestatic compounds. A second model was set up focusing only on predicting cholestasis or non-hepatotoxicity of all cholestatic and non-hepatotoxic compounds in the training and validation set. In an attempt to train and validate with two independent sets, the PAM model was trained using the cholestatic and non-hepatotoxic compounds of the original training set and validated using the cholestatic and non-hepatotoxic compounds of the initial validation set (Table 6). This resulted in an accuracy of 78% in this validation set based on 16 genes. However, it has to be noted that training of this PAM model was only done using 3 cholestatic and 3 non-hepatotoxic compounds. To reduce the risk of overtraining the model and only find compound-specific changes, a new PAM model was setup with a combined dataset of the cholestatic and non-hepatotoxic compounds of the training and validation set. Cholestasis could be classified with an accuracy of 93% based on 12 genes. These results are based on a validation using the same data that was used to train the PAM model, thus in follow up studies, validation of these classifiers for cholestasis with an independent data set is needed. 6 of the found genes, AARS, ASNS, CTH, DDIT3, DNAJB9 and INHBE, are associated with ER stress and the UPR. The MTHFD2 gene was also found as classifier for hepatotoxicity and is associated with folate-mediated one-carbon metabolism and disruption hereof is associated with many pathologies. [43] The other 5 genes are associated with several GO terms indicative of a toxicological response, such as DNA damage response.

One of the hallmarks of cholestasis is the accumulation of bile acids in serum and hepatocytes. Bernstein et al. showed that the bile salt deoxycholate activates promoters of genes associated with DNA damage and protein malfolding [44] and accumulation of bile acids in hepatocytes may thus induce oxidative stress and the UPR. Recently, Vinken et al. developed an Adverse Outcome Pathway (AOP) for cholestasis and proposed that this AOP serves as the basis for new *in vitro* tests and biomarkers of drug-induced cholestasis. [45] Although important cellular effects such as mitochondrial permeability pore (MPP) and oxidative stress are included in this AOP, no information on ER stress or UPR is added. ER stress and UPR can lead to Ca^{2+} release into the cytosol and this Ca^{2+} can be taken up by mitochondria leading to opening of the MPP, triggering oxidative stress and ultimately apoptosis. [38] The results from this manuscript suggest that ER stress and the UPR may be important early cellular effects in drug-induced hepatotoxicity and cholestasis and should therefore be further investigated in the search for new *in vitro* tests and biomarkers.

Conclusions

We were able to prove the principle that transcriptomic analyses of HepG2 cells can be used to classify hepatotoxicity. Classifiers selected for classification of hepatotoxicity and cholestasis indicate that endoplasmic reticulum stress and the unfolded protein response may be important cellular effects of drug-induced liver injury. However, the number of compounds in both the training and validation sets should be increased to further validate the prediction.

Supporting information

The data sets supporting the results of this article are included within the article and additional files or are available in the NCBI's gene expression omnibus. [46] The transcriptomics data are accessible through GEO series accession number GSE51952. Additional file 1 and 2 consist of the log2 fold change values of the 36 genes selected for classification of hepatotoxicity and the 12 genes selected for classification of cholestasis, respectively. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Table 1. *Compounds of the training set with their corresponding class, subclass and the IC_{20} concentrations measured after 24 hours.*

Training set

Compound	*Abbreviation*	*CAS no*	*Solvent*	*Class*	*Subclass*	*Dose (μM)*
Acetaminophen	*APAP*	*103-90-2*	*DMSO*	*Hepatotoxic*	*Necrosis*	*10000*
Adefovir	*ADF*	*106941-25-7*	*DMSO*	*Non-hepatotoxic*	*Non-hepatotoxic*	*50*
Amiodarone	*AM*	*1951-25-3*	*DMSO*	*Hepatotoxic*	*Steatosis*	*15*
Chlorpromazine	*CLP*	*50-53-3*	*DMSO*	*Hepatotoxic*	*Cholestasis*	*25*
Cyclosporin A	*CsA*	*59865-13-3*	*DMSO*	*Hepatotoxic*	*Cholestasis*	*20*
Diclofenac	*DIC*	*15307-86-5*	*PBS*	*Hepatotoxic*	*Necrosis*	*500*
D-mannitol	*Dman*	*69–65–8*	*PBS*	*Non-hepatotoxic*	*Non-hepatotoxic*	*2000*
Ethinyl Estradiol	*EE*	*57-63-6*	*DMSO*	*Hepatotoxic*	*Cholestasis*	*90*
Isoniazid	*ISO*	*54-85-3*	*DMSO*	*Hepatotoxic*	*Necrosis*	*2000*
Lithium Carbonate	*LiC*	*554-13-2*	*PBS*	*Non-hepatotoxic*	*Non-hepatotoxic*	*2000*
Paraquat	*PAQ*	*1910-42-5*	*PBS*	*Hepatotoxic*	*Necrosis*	*120*
Tetracycline	*TET*	*60-54-8*	*DMSO*	*Hepatotoxic*	*Steatosis*	*2000*
Valproic Acid	*VPA*	*99-66-1*	*PBS*	*Hepatotoxic*	*Steatosis*	*2000*

Table 2. *Compounds of the validation set with their corresponding class, subclass and the IC_{20} concentrations measured after 72 hours.*

Validation set

Compound	*Abbreviation*	*CAS no*	*Solvent*	*Class*	*Subclass*	*Dose (μM)*
17-beta Estradiol	*E2*	*50-28-2*	*DMSO*	*Hepatotoxic*	*Cholestasis*	*30*
Azathioprine	*AZA*	*446-86-6*	*DMSO*	*Hepatotoxic*	*Cholestasis*	*250*
Cisplatin	*cisPt*	*15663-27-1*	*PBS*	*Hepatotoxic*	*Necrosis*	*20*
Clonidine	*Clo*	*4205-90-7*	*DMSO*	*Non-hepatotoxic*	*Non-hepatotoxic*	*500*
Cyclophosphamide	*CP*	*50-18-0*	*PBS*	*Hepatotoxic*	*Cholestasis*	*2000*
Cyclosporin A	*CsA*	*59865-13-3*	*DMSO*	*Hepatotoxic*	*Cholestasis*	*3*
D-mannitol	*Dman*	*69–65–8*	*PBS*	*Non-hepatotoxic*	*Non-hepatotoxic*	*250*
Mitomycin C	*MMC*	*50-07-7*	*DMSO*	*Non-hepatotoxic*	*Non-hepatotoxic*	*0.2*
Nifedipine	*Nfe*	*21829-25-4*	*DMSO*	*Hepatotoxic*	*Cholestasis*	*106.25*
Phenobarbital	*Phb*	*50-06-6*	*DMSO*	*Hepatotoxic*	*Cholestasis*	*1000*
Tolbutamide	*Tb*	*64-77-7*	*DMSO*	*Hepatotoxic*	*Cholestasis*	*2000*

Table 3. *Results of the prediction of hepatotoxicity for the compounds in the training set and validation set.*

Training set as validation		**Validation set as validation**	
Accuracy	*92%*	*Accuracy*	*91%*
Sensitivity	*90%*	*Sensitivity*	*88%*
False negative rate	*10%*	*False negative rate*	*12%*
Specificity	*100%*	*Specificity*	*100%*
False positive rate	*0%*	*False positive rate*	*0%*
Positive predictive value	*100%*	*Positive predictive value*	*100%*
Negative predictive value	*75%*	*Negative predictive value*	*75%*

Table 4. *Genes selected by PAM for prediction of hepatotoxicity versus non-hepatotoxicity.*

	Entrez Gene ID	Gene Symbol	Description
Up-regulated genes	16	AARS	Aminoacyl tRNA synthetase
	113	ADCY7	Adenylate cyclase type 7
	1646	AKR1C2	Aldo-keto reductase family 1 member C 2
	440	ASNS	Asparagine synthetase
	875	CBS	Cystathionine beta synthase
	9236	CCPG1	Cell cycle progression 1
	1491	CTH	Cystathionine gamma-lyase
	57834	CYP4F11	Cytochrome P450, family 4, subfamily F, polypeptide 11
	1649	DDIT3	DNA damage-inducible transcript 3
	1978	EIF4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
	9569	GTF2IRD1	General transcription factor II-I repeat domain-containing protein 1
	83729	INHBE	Inhibin, beta E
	9388	LIPG	Endothelial lipase
	10797	MTHFD2	Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase
	283131	NEAT1	Nuclear paraspeckle assembly transcript 1
	4758	NEU1	Sialidase 1 (lysosomal sialidase)
	29095	ORMDL2	ORM1 like protein 2
	80336	PABPC1L	Poly(A) binding protein, cytoplasmic 1-like
	140809	SRXN1	Sulfiredoxin 1
	51175	TUBE1	Tubulin, epsilon 1
	100507213	USP6NL-IT1	USP6NL intronic transcript 1
	11260	XPOT	Exportin-T
Down-regulated genes	57291	DANCR	Differentiation antagonizing non-protein coding RNA
	1906	EDN1	Endothelin 1
	9837	GINS1	DNA replication complex GINS protein PSF1
	220296	HEPACAM	Hepatic and glial cell adhesion molecule
	3183	HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2
	100505650	LOC100505650	Uncharacterized LOC100505650
	4153	MBL2	Mannose-binding lectin
	5019	OXCT1	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1
	5422	POLA1	DNA polymerase alpha catalytic subunit
	6157	RPL27A	60S Ribosomal protein L27a
	10590	SCGN	Secretagogin
	8436	SDPR	Serum deprivation-response protein
	10772	SRSF10	Serine/arginine-rich splicing factor 10
	57473	ZNF512B	Zinc finger protein 512B

Table 5. *Relevant Gene Ontology terms associated with the selected features for classification of hepatotoxic compounds versus non-hepatotoxic compounds.*

	Gene Symbol	Gene Ontology Terms (Biological processes)
Up-regulated genes	AARS	Protein folding; Unfolded protein response
	ADCY7	Intracellular signal transduction; Transmembrane transport
	AKR1C2	Positive regulation of cell proliferation; Bile acid binding
	ASNS	Activation of signaling protein activity involved in unfolded protein response
	CBS	Negative regulation of apoptotic process
	CCPG1	Cell cycle
	CTH	Transsulfuration; Endoplasmic reticulum unfolded protein response
	CYP4F11	Xenobiotic metabolic process; Arachidonic acid or fatty acid metabolism
	DDIT3	Activation of signaling protein activity involved in unfolded protein response
	EIF4EBP1	G1/S transition of mitotic cell cycle; Positive regulation of mitotic cell cycle
	GTF2IRD1	Regulation of transcription, DNA-dependent
	INHBE	No Gene Ontology terms were found
	LIPG	Lipid metabolic process; Cholesterol homeostasis; Cell proliferation
	MTHFD2	One-carbon metabolic process; Folic acid-containing compound biosynthetic process
	NEAT1	No Gene Ontology terms were found
	NEU1	Sphingolipid metabolic process
	ORMDL2	Ceramide metabolic process; Endoplasmic reticulum
	PABPC1L	RNA binding
	SRXN1	Response to oxidative stress
	TUBE1	Centrosome cycle; Structural constituent of cytoskeleton
	USP6NL-IT1	No Gene Ontology terms were found
	XPOT	Intracellular protein transport; tRNA binding
Down-regulated genes	DANCR	Differentiation
	EDN1	Positive regulation of cell proliferation; Calcium-mediated signaling
	GINS1	Mitotic cell cycle; DNA strand elongation involved in DNA replication
	HEPACAM	Cell cycle arrest
	HNRNPC	mRNA binding
	LOC100505650	No Gene Ontology terms were found
	MBL2	Response to oxidative stress; Complement activation; Calcium-dependent protein binding
	OXCT1	Cellular lipid metabolic process; Response to drug
	POLA1	DNA replication; Cell proliferation
	RPL27A	Translation
	SCGN	Calcium ion binding
	SDPR	Phosphatidylserine binding
	SRSF10	Unfolded protein binding; Regulation of transcription, DNA-dependent
	ZNF512B	Regulation of transcription, DNA-dependent

Table 6. *Results of the prediction of cholestasis and non-hepatotoxicity for the cholestatic and non-hepatotoxic compounds in the training and validation set.*

	Training set	Validation set	Combination set
Accuracy	*100%*	*78%*	*93%*
Sensitivity	*100%*	*67%*	*89%*
False negative rate	*0%*	*33%*	*11%*
Specificity	*100%*	*100%*	*100%*
False positive rate	*0%*	*0%*	*0%*
Positive predictive value	*100%*	*100%*	*100%*
Negative predictive value	*100%*	*60%*	*86%*

Table 7. *Results of the prediction of cholestasis and non-hepatotoxicity for the combined set of cholestatic and non-hepatotoxic compounds of the training and validation set.*

Table 8. *Genes selected by PAM for prediction of cholestasis versus non-hepatotoxicity.*

Entrez Gene ID	Gene Symbol	Description
16	*AARS*	*Aminoacyl tRNA synthetase*
440	*ASNS*	*Asparagine synthetase*
1491	*CTH*	*Cystathionase*
1649	*DDIT3*	*DNA-damage-inducible transcript 3*
4189	*DNAJB9*	*DnaJ (Hsp40) homolog, subfamily B, member 9*
5106	*PCK2*	*Phosphoenolpyruvate carboxykinase 2*
5450	*POU2AF1*	*POU class 2 associating factor 1*
6286	*S100P*	*S100 calcium binding protein P*
7298	*TYMS*	*Thymidylate synthetase*
10797	*MTHFD2*	*Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2*
83729	*INHBE*	*Inhibin, beta E*
144455	*E2F7*	*E2F transcription factor 7*

Table 9. *Relevant Gene Ontology terms associated with the selected features for classification of cholestatic compounds versus non-hepatotoxic compounds.*

Gene Symbol	Gene Ontology Terms (Biological processes)
AARS	*Protein folding; Unfolded protein response*
ASNS	*Activation of signaling protein activity involved in unfolded protein response*
CTH	*Transsulfuration; Endoplasmic reticulum unfolded protein response*
DDIT3	*Activation of signaling protein activity involved in unfolded protein response*
DNAJB9	*Endoplasmic reticulum unfolded protein response*
PCK2	*Carbohydrate metabolic process; Gluconeogenesis*
POU2AF1	*Humoral immune response; Regulation of transcription, DNA-dependent*
S100P	*Calcium-dependent protein binding; Response to organic substance*
TYMS	*Response to drug; DNA replication and repair*
MTHFD2	*One-carbon metabolic process; Folic acid-containing compound biosynthetic process*
INHBE	*No Gene Ontology terms were found*
E2F7	*Hepatocyte differentiation; DNA damage response*

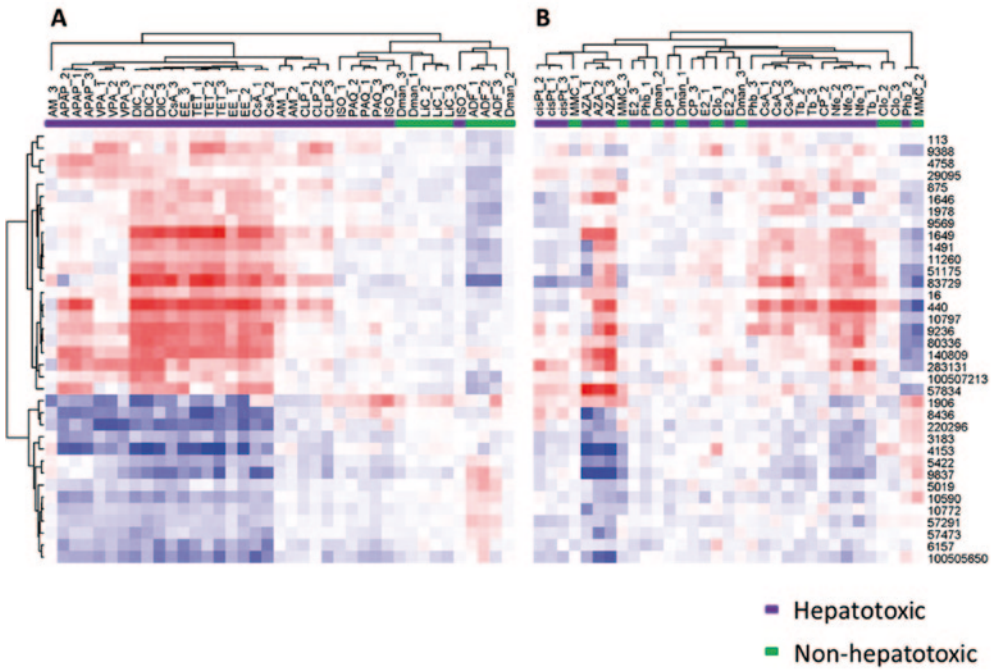

Figure 1. *Visualization of gene expression patterns by hierarchical cluster analyses of the 36 genes for the training set (A) and validation set (B) based on the Log2 fold changes between compound and solvent control.*

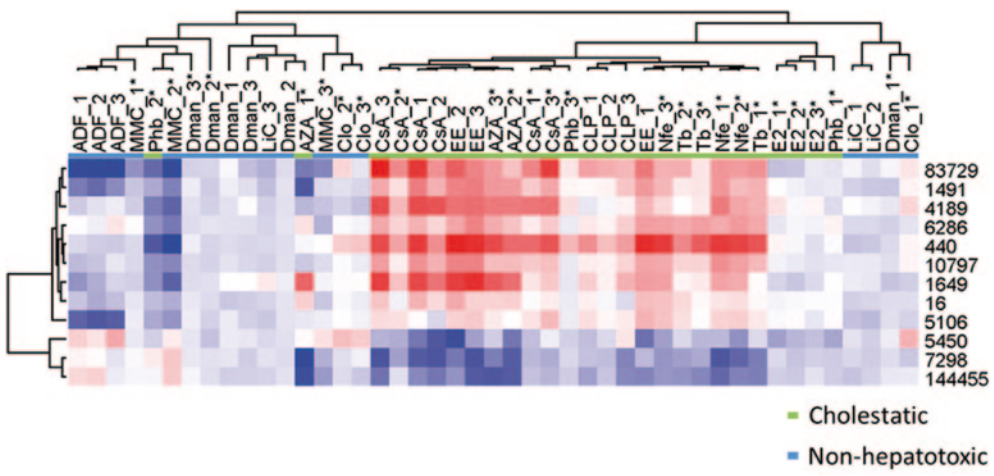

Figure 3. *Visualization of gene expression patterns by hierarchical cluster analysis of the 12 genes for the cholestatic and non-hepatotoxic compounds based on the Log2 fold changes between compound and solvent control (* compounds from the validation set).*

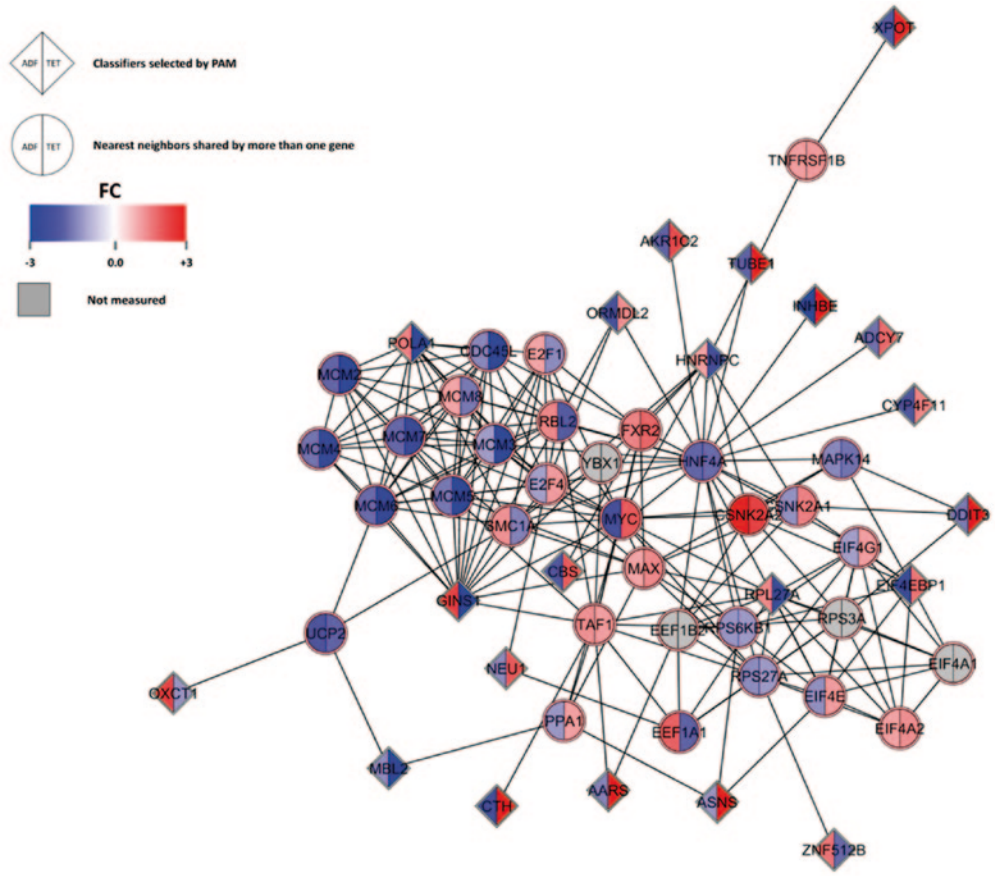

Figure 2. *Network created with Cytoscape based on interactions of the genes selected by PAM (diamonds) and their nearest neighbors shared by more than one gene (circles) and visualized using the fold changes after treatment with the hepatotoxic compound Tetaracycline (TET) and non-hepatotoxic compound Adefovir (ADF).*

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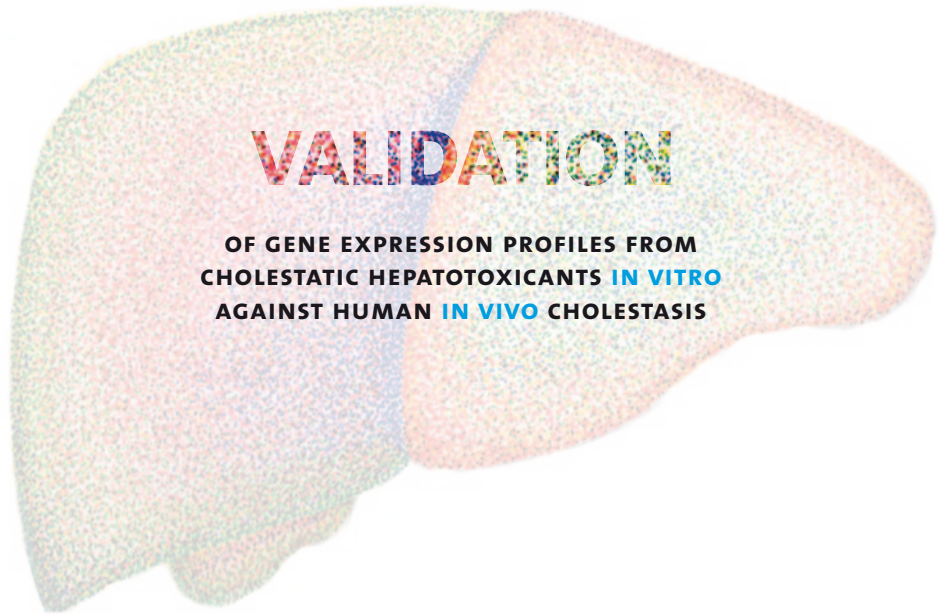
Chapter 3



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Abstract

Drug-induced liver injury remains the most common cause of acute liver failure and a frequently indicated reason for withdrawal of drugs. Cholestasis is one of the most severe manifestations of drug-induced hepatotoxicity. Intracellular bile accumulation via the inhibition of transport proteins has been suggested to be the main underlying mechanism of drug-induced cholestasis. There is an increasing demand for liver models better capable of predicting drug-induced cholestasis in humans. We here aimed to benchmark 'omics-derived mechanistic data from three *in vitro* models for parenchymal liver function, for the investigation of drug-induced cholestasis against omics data from cholestatic patients.

Transcriptomic changes in HepG2 cells, primary mouse hepatocytes and primary human hepatocytes exposed to Cyclosporin A, Chlorpromazine and Ethinyl Estradiol were analyzed using microarrays. In order to find an *in vitro* fingerprint of drug-induced cholestasis, the overlapping differentially expressed genes were selected in each model. 151 genes were differentially expressed in all treatments in HepG2 cells of which 13 were also differentially expressed in human cholestasis. The overlap between drug-induced transcriptomic responses in primary mouse hepatocytes and primary human hepatocytes appeared limited and no overlapping genes with *in vivo* cholestasis were found. Gene ontology terms associated with the 13 overlapping gene expressions in *in vivo* cholestasis and in drug-induced cholestasis in HepG2 cells indicated the involvement of the unfolded protein response in cholestasis. The limited overlap between expression profiles of the different compounds however suggests different mechanisms. Thereupon, a previously published pathway for drug-induced cholestasis was used to map the drug-induced transcriptomic modifications involved in bile homeostasis, and compare those to gene expression changes in human cholestasis.

Indications of an adaptive response to prevent and reduce intracellular bile accumulation were observed *in vivo* as well as *in vitro*. Furthermore, drug-specific changes on the transcription of several important bile salt secreting and conjugating genes were found. These drug-induced changes may result in intracellular accumulation of bile constituents and may be indicative of their cholestatic properties.

Introduction

The liver is the most important organ in the metabolism and excretion of drugs and toxins, thus making it also vulnerable for adverse reactions. Drug-induced liver injury (DILI) is the most common cause of acute liver failure and represents a frequently indicated reason for withdrawal of drugs in clinical trials or even after admittance onto the market. Cholestasis having a high mortality rate, is one of the most severe manifestations of DILI and may account for 16% or up to 50% of all cases. [1] Cholestasis is characterized by the accumulation in the liver or bile ducts of substances normally excreted in the bile such as bile salts, cholesterol and drug metabolites, due to inhibition of canalicular transporter function or impaired bile flow.

One of the most important transporter proteins for canalicular secretion of conjugated bile salts in hepatocytes is the Bile Salt Export Pump (BSEP). Vinken et al. published an Adverse Outcome Pathway (AOP) describing the onset of cholestasis due to the accumulation of bile salts resulting from the inhibition of the BSEP. [2] However, not all cholestatic compounds inhibit the BSEP, which indicates that the mechanisms underlying drug-induced cholestasis still needs further investigation. Yuryev et al. published a drug-induced cholestasis pathway which includes important genes involved in the intake, synthesis, conjugation and secretion of bile salts and the influence of several drugs on these genes. [3] Furthermore, this pathway includes the nuclear receptors Farnsoid X Receptor (FXR), Pregnane X Receptor (PXR), Vitamin D Receptor (VDR) and Constitutive Androstane Receptor (CAR) which act as sensors of bile salts and are involved in two feedback loops.

Since only approximately 60% of predictions of the repeated dose rodent bioassay used for toxicity screening are relevant for human, great effort is put in finding *in vitro* alternatives which are capable of providing better predictive models. [4] It is shown that comparing *in vitro* results to relevant *in vivo* datasets may increase the biological plausibility of *in vitro* findings. [5-6] Therefore, it would be of relevance to compare drug-induced cholestasis in *in vitro* models to drug-induced cholestasis in patients. However, large-scale *in vivo* transcriptomics experiments of drug-induced cholestasis in humans are not available. Therefore, in this study, microarray data of liver samples of patients suffering from extrahepatic cholestasis were analyzed, in order to identify a transcriptomic fingerprint of cholestasis *in vivo*.

The gold standard for *in vitro* alternatives for human liver is presented by primary human hepatocytes (PHH). [7] For the present study, transcriptomic data of PHH exposed to cholestatic compounds were derived from the Open TG-GATEs database. However, in general, human donors are scarce and there is a high inter-individual variation, making it hard to use these cells in large-scale screening experiments. Since most drug toxicity screenings are performed in rodents and the use of inbred mouse strains reduces the inter-individual variation, primary mouse hepatocytes (PMH) which are readily available, are also suggested as *in vitro* alternative. [8] However, the extrapolation of mouse *in vitro* results to the human *in vivo* situation might be complicated. Therefore, a third alternative may be presented by HepG2 cells, a human hepatoblastoma-derived cell-line, which is frequently used as an *in vitro* liver model and allows the classification of hepatotoxicants. [9] However, the actual relevance of such alternative *in vitro* models for predicting liver toxicity in drug-treated patients still remains to be evaluated.

In this study, we thus validated the transcriptomic changes in PHH, PMH and HepG2 cells induced by cholestatic compounds, against the transcriptomic fingerprint derived from liver samples from patients suffering from extrahepatic cholestasis (manuscript in preparation). For this purpose, we analyzed cellular responses to treatment with three prototypical compounds, which are known to induce cholestasis in humans, namely Cyclosporin A (CsA), Chlorpromazine (CPZ) and Ethinyl Estradiol (EE).

Materials and methods

Chemicals

Modified Eagle's medium (MEM) plus glutamax, fetal calf serum (FCS), non-essential amino acids, sodium pyruvate, penicillin/streptomycin, insulin and Hanks' calcium- and magnesium-free buffer were purchased from Invitrogen (Breda, The Netherlands). Glucagon, hydrocortisone (water soluble), collagenase type IV, dimethylsulfoxide (DMSO), Trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Cyclosporin A, Chlorpromazine, Ethinyl Estradiol, NaCl, $NaHCO_3$, KCl, KH_2PO_4, $MgSO_4$, glucose, and $CaCl_2$ were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). Collagen type I Rat Tail was obtained from BD Biosciences (Bedford, MA, USA). The Trizol reagent and the RNeasy mini kit were from Qiagen Westburg (Leusden, The Netherlands).

Human in vivo data

Microarray data of cholestatic liver samples and control liver samples were obtained from a different study (manuscript in preparation) using samples from previous published research. [10] In short, the respective protocols of the studies were approved by the local Medical Ethical Committee and the patients gave their informed consent. Samples were derived from 9 perioperative liver biopsies from patients with a pancreatic or periampullary malignancy and subsequent obstructive jaundice (cholestatic group) and from 9 nonjaundiced patients with a pancreatic malignancy or undergoing liver resection (control group). Liver samples were collected in RNAlater and stored at -80°C until RNA isolation. Isolated RNA was hybridized to Agilent SurePrint G3 Human Gene Expression 8x60K v2 Microarrays and scanned using an Affymetrix GeneArray scanner.

Primary human hepatocytes data

Data of primary human hepatocytes exposed to CsA, CPZ and EE were retrieved from the Open TG-GATEs database (http://toxico.nibio.go.jp). In total 22 Affymetrix CEL-files were collected. Hepatocytes were exposed for 24 hours *in duplo* to the compounds and 0.5% DMSO as solvent control. The used concentrations to compare to exposures in the other cell lines were: 20 µM for CPZ, 6 µM for CsA and 15 µM for EE.

HepG2 data

In total 72 Affymetrix CEL-files of HepG2 cells exposed to CsA, CPZ and EE and matching controls were collected from previously published data. [9] HepG2 cells were exposed for 24 hours to the

IC_{20} of 24 hours. Concentrations were as follows: CsA, 20 µM; CPZ, 25 µM; EE, 90 µM.

Primary mouse hepatocytes data

The animal studies were approved by the Animal Ethical Committee of the Maastricht University, The Netherlands (approval number: 2011-108). Adult male C57BL/6 mice, weighing 20–25 grams, were obtained from Charles River GmbH, Sulzfeld, Germany. Animals were housed in macrolon cages with sawdust bedding at 22°C and 50–60% humidity. Food and tap water were available *ad libitum,* and the light cycle was 12 hours light/12 hours dark. Isolation and culture of primary mouse hepatocytes were performed as described before. [8] After the recovery period, cells were exposed to culture medium containing Chlorpromazine, Ethinyl Estradiol or 0.5% v/v DMSO as a vehicle control. Based on the IC_{20} values for 24 and 48 hours exposure, which were determined by the MTT reduction method [11], the hepatocytes were exposed as follows: 0.5% v/v DMSO as a vehicle control, 20 µM CPZ and 100 µM EE for 24 hours. Isolated RNA was labeled and hybridized to Affymetrix Mouse Genome 430 2.0 arrays and scanned using an Affymetrix GeneArray scanner.

Data of primary mouse hepatocytes exposed to CsA were derived from a previous study. [12] PMH were exposed for 24 to 50 µM CsA, which is the IC_{20} value for 24 hours. In total, the primary mouse hepatocytes set comprised 42 arrays.

Data normalization and preprocessing

Quality of Agilent arrays was being determined applying an in-house developed pipeline in R version 3.0.2 R Development Core. [13] Probes that were flagged by this pipeline were left out for further processing. Arrays were quantile normalized and replicate probes were summarized by taking the median. Probes that were present in 75% of all arrays were included for further processing. Remaining probes were reannotated to EntrezGeneIDs using Agilent annotation file (date: 2012-06-28).

Quality of Affymetrix CEL-files was assessed by the arrayanalysis.org webservice. [14] All arrays were determined of high quality. Probesets were reannotated to EntrezGene IDs using Brainarray's custom CDF version 17.1.0, as described by Dai et al. [15] and normalized using the RMA method combined with MAS5-PMA-calling. [16] Only probes that were present or marginal in at least 2 out of 3 replicates in all experimental groups were selected for further analysis, and their intensities were transformed to LogRatios relative to their matching controls.

Statistical analyses

Features that passed the Agilent QC (Human *in vivo*: 17581) and the Affymetrix PMA-criteria (HepG2: 11381; PMH: 10436; PHH: 10800) were used as input for analysis of differentially expressed genes (DEGs) using the BioConductor package LIMMA version 3.18.3. [17] First, per cell line a linear model was fitted to the expression data, whereby replicate information (pairing) was treated as random effect. Subsequently, contrasts were defined that estimated the compound effect over DMSO controls. A moderated t-test was executed to find DEGs based on a combination of the following criteria: I) a P-value <0.05, II) an average absolute fold change (FC) of 1.5 or higher (i.e. average log2 ratio of < -0.58 or >0.58).

Results

Comparison of transcriptomic effects

In order to find a transcriptomic fingerprint of cholestasis *in vivo*, differences in mRNA expression of liver biopsies from patients with and without cholestasis were analyzed and compared (Table 1). In total, 1245 genes appeared significantly up-regulated and 1097 genes were down-regulated in the cholestasis samples compared to the healthy controls.

The transcriptomic responses of three *in vitro* models, HepG2 cells, PMH and PHH, to three cholestasis-inducing compounds, CsA, CPZ and EE, were analyzed. Exposure for 24 hours to these cholestatic compounds was analyzed in all *in vitro* models and this time point was therefore used to compare the drug-induced transcriptomic changes. Table 1 summarizes the number of differentially expressed genes (DEGs) for the three treatments in all three *in vitro* models. Of the three compounds, CsA treatment induced the largest number of transcriptional changes in all *in vitro* models. Overall, the highest number of DEGs was found in HepG2 cells. The PMH model demonstrated a similar number of DEGs as the HepG2 cells after CsA treatment; however, the transcriptomic response after treatment with CPZ and EE was much lower. In general, the PHH model showed the lowest number of DEGs after treatment with the cholestatic compounds.

In order to find cholestasis-specific changes rather than compound-specific changes, the overlap of DEGs between the three compounds was analyzed using Venn Diagrams. In HepG2 cells, 68 genes were differentially up-regulated and 83 genes were differentially down-regulated in all three treatments (Figure 1 A and B). Two DEGs were up-regulated and two down-regulated in all three treatments in the PMH model (Figure 1 C and D) and only one gene was differentially up-regulated in all treatments in the PHH model, where no overlapping down-regulated genes were found (Figure 1 E and F). The genes found to have a significantly changed expression into the same direction in all three treatments in the PMH and PHH models, were not differentially expressed, nor differentially expressed in the opposite direction, in the human *in vivo* samples. By contrast, HepG2 data did demonstrate overlap with the human *in vivo* data. Figure 2 shows the overlap of up-regulated DEGs (Figure 2 A) and down-regulated DEGs (Figure 2 B) between *in vivo* cholestasis samples and HepG2 cells exposed to the three cholestatic compounds. 12 genes were found to be differentially up-regulated both in *in vivo* cholestasis and in HepG2 cells exposed to cholestatic compounds. The SNRNP25 gene was found differentially down-regulated in both groups (Table 2), which is involved in mRNA processing. The 12 up-regulated genes are involved in several processes including lysosomal processes and the Endoplasmic Reticulum (ER) stress response.

Overall, the overlap between DEGs in *in vivo* cholestasis and DEGs after treatment with the three cholestatic compounds *in vitro* is limited and only DEGs in HepG2 cells showed the same directionality as DEGs in *in vivo* samples.

Pathway mapping

A previously published drug-induced cholestasis pathway was used to map significant

transcriptomic changes in *in vivo* cholestasis and after *in vitro* treatment of HepG2 cells, PMH and PHH with the three cholestatic compounds (Table 3). The mRNA level of the SLCO1B1 gene, which is involved in the uptake of unconjugated bile salts, appeared significantly down-regulated in the human cholestasis samples which thus may prevent further intracellular accumulation of bile salts. However, no significant changes in mRNA levels of this gene were detected in HepG2 cells and PHH. In PMH, no homologue for this gene was found and could therefore not be assessed. The SLC10A1 gene coding for NTCP is involved in the uptake of conjugated bile salts but appeared not significantly affected in *in vivo* cholestasis, where CsA treatment in PMH and CPZ treatment in PHH significantly down-regulate SLC10A1 mRNA level which may thus decrease the uptake of bile salts. Expression of this gene could not be demonstrated in HepG2 cells.

CYP7A1 and CYP27A1 are the rate-limiting enzymes in the synthesis of bile salts and mRNA levels of CYP7A1 were drastically down-regulated in *in vivo* cholestasis. CYP27A1 expression was down-regulated in PMH after treatment with CsA. CYP7A1 expression was not measured in PHH.

CYP3A4, BAAT and SULT2A1 are involved in the hydroxylation and conjugation of bile salts and toxic compounds. BAAT and CYP3A4 are down-regulated in *in vivo* cholestasis. SULT2A1 is down regulated in HepG2 cells after treatment with CsA and EE, but up-regulated after CPZ treatment. In PMH, BAAT is differentially expressed after treatment with CsA and EE, however, after CsA treatment expression is down-regulated and after EE treatment expression is up-regulated. In PHH, SULT2A1 is significantly up-regulated after CsA treatment and CYP3A4 is significantly up-regulated after treatment with CPZ and EE. CYP3A4 was not measured in HepG2 cells and no homologue was found in PMH and SULT2A1 was not assessed in PMH.

7 genes involved in the basolateral or canalicular secretion of bile salts are included in the drug-induced cholestasis pathway and three of these are significantly up-regulated in *in vivo* cholestasis, namely OSTα, OSTβ and ABCB11 (BSEP). ABCC4 expression was not measured in the *in vivo* cholestasis samples. In HepG2 cells, treatment with CsA and EE resulted in a down-regulation of OSTα and an up-regulation of ABCC2, ABCC3 and ABCB1. Furthermore, EE treatment of HepG2 cells up-regulated the expression of OSTβ and ABCC4. ABCB11 expression was not assessed in HepG2 cells. In PMH ABCB11, ABCB1 and ABCC3 were up-regulated after treatment with EE. However, an opposite effect on these genes was found after treatment with CsA. Expression levels of OSTα and OSTβ were not assessed in PMH. In PHH, only one of the genes involved in secretion was differentially expressed, namely OSTα, which was down-regulated after treatment with CsA.

The drug-induced cholestasis pathway includes four nuclear receptors, NR1H4 (FXR), VDR, NR1I2 (PXR) and NR1I3 (CAR). PXR and CAR are down-regulated in *in vivo* cholestasis. In HepG2 cells, FXR is up-regulated after treatment with CsA and CPZ. CAR expression was not assessed in HepG2 cells. CsA treatment resulted in a down-regulation of FXR and PXR in PMH. Expression levels of VDR and CAR were not measured in PMH. No nuclear receptors were differentially expressed in PHH after treatment with cholestatic compounds.

The DEGs of the established drug-induced cholestasis pathway *in vivo* suggest an adaptive response to cholestasis, where the uptake and synthesis of bile salts is down-regulated and

their secretion up-regulated. However, not all genes in the drug-induced cholestasis pathway were differentially expressed in the patient samples. Although the *in vitro* data shows some similarities to the *in vivo* data, overall no clear response of these pathway-specific genes is observed in any of the *in vitro* models.

Discussion

In this study we aimed to investigate the transcriptomics fingerprint of cholestasis and we compared *in vitro* cholestasis induced by drugs to human cholestasis. Since samples of *in vivo* drug-induced cholestasis were not available, we evaluated data derived from liver biopsies from patients suffering from non-drug-induced cholestasis, in this case extra-hepatic cholestasis caused by a pancreatic tumor. In these patients, the pancreatic tumor obstructs the flow of bile through the common bile ducts towards the duodenum, causing accumulation of bile in the bile ducts, the liver and plasma. [10] Cholestasis *in vivo* will activate an adaptive response in which the uptake and synthesis of bile salts in hepatocytes is down-regulated and the hepatocellular secretion is up-regulated in order to prevent further accumulation of bile salts. [18] This adaptive response is to some degree also present in the *in vivo* samples of extra-hepatic cholestasis and is visualized in Table 3 presenting the drug-induced cholestasis pathway. The uptake of bile salts is down-regulated in cholestatic patients via the down-regulation of the solute carrier organic anion transporter family member 1B1. CYP7A1 is the rate-limiting enzyme in the classic pathway of bile salt synthesis. [19] The CYP7A1 gene appears differentially down-regulated in cholestatic patients in this study, which implies a down-regulation of bile salt synthesis. Furthermore, the transcription of the hepatocellular export proteins OST-α, OST-β and the BSEP was significantly up-regulated in these *in vivo* samples, which may be an indication of up-regulated bile salt secretion. However, the nuclear receptors that are thought to control the adaptive response after activation by bile salts are not differentially up-regulated in the studied human cholestatic samples, PXR and CAR even being down-regulated. Furthermore, BAAT and CYP3A4, which are involved in preventive conjugation of bile salts, are also down-regulated. Schaap et al. suggested that FGF19, normally produced by the small intestine but ectopically overexpressed in the human cholestatic liver, may be involved in the adaptive response, including the down-regulation of CYP7A1 expression. [10] Overall, an adaptive response is observed in the patient samples using the drug-induced cholestasis pathway, which confirms the involvement of the genes in this pathway in *in vivo* cholestasis. However, not all genes of the drug-induced cholestasis pathway are significantly changed in the patient samples. Therefore, further validation of this pathway, using *in vivo* data on drug-induced cholestasis, is necessary.

In order to create a transcriptomic fingerprint of drug-induced cholestasis, three liver cell models were exposed to three cholestasis-inducing compounds. In an attempt to select cholestasis-specific DEGs, the overlap between DEGs in all three treatments was selected. 151 genes were differentially expressed into the same direction in all three treatments in HepG2 cells, of which 13 were also significantly changed in *in vivo* cholestasis. These overlapping genes are involved in several processes, including cell proliferation and metabolism. Furthermore, genes involved in ER stress and in the unfolded protein response, were differentially expressed.

In vitro classification of cholestatic compounds previously indicated that the UPR may be an important cellular effect in drug-induced hepatotoxicity.[9] However, in the other two cell models, only low numbers of overlapping DEGs were found, which were not differentially expressed *in vivo*. Overall, these low numbers of overlapping DEGs suggest different mechanisms for the three cholestatic drugs.

Cyclosporin A is a known inhibitor of the BSEP and several other ATP-dependent export carriers, which is suggested to be the mechanism behind CsA-induced cholestasis.[20-21] The inhibition of the BSEP could not be assessed in HepG2 cells since expression of the BSEP is low in these cells and the expression values of the ABCB11 gene did not pass the PMA criteria.[22-23] The ABCB11 gene was significantly down-regulated in PMH, but no significant change was found in PHH. In HepG2 cells and PHH, CsA treatment down-regulated OST-α, which may point to CsA-induced accumulation of bile constituents. Three other proteins involved in secretion were differentially up-regulated in HepG2 cells after CsA treatment, ABCC2, ABCC3 and ABCB1. In combination with the observed up-regulation of the FXR, this may indicate an adaptive response of HepG2 cells to CsA-induced toxicity. However, ABCC3 and ABCB1 were significantly down-regulated after CsA-treatment in PMH, suggesting differences in CsA-induced toxicity between human and mouse cells. CsA exposure in PHH resulted in an up-regulation of SULT2A1, which catalyzes the sulfate conjugation of many substrates, including bile salts, which increases their solubility and excretion.[24] Although the three cell models show differences in differentially expressed genes after CsA treatment, indications for significant changes of expression of CsA-specific genes and genes involved in the cholestasis-related adaptive response were observed in all cell models.

CPZ treatment only resulted in changes in PHH and HepG2 cells when evaluating its impact on the *a priori* defined cholestasis pathway. SLC10A1 involved in the uptake of bile salts, was down-regulated in PHH, which may indicate an adaptive response to CPZ-induced cholestasis in PHH. Furthermore, CYP3A4 was up-regulated in PHH after CPZ treatment. CYP3A4 is involved in the metabolism of CPZ and up-regulation of this enzyme suggests that the PHH up-regulate the breakdown of CPZ and accumulated bile salts.[25] Down-regulation of SLC10A1 and up-regulation of CYP3A4 was also observed in HepaRG cells after CPZ treatment.[26] Anthérieu et al. report CPZ-induced ROS formation which resulted in taurocholic acid accumulation as an early response and an adaptive response to cholestasis after 24 hours exposure to CPZ. CPZ exposure of HepG2 cells resulted in an up-regulation of FXR and SULT2A1. FXR is up-regulated as a response to accumulating bile salts and activates the adaptive response including increased bile salt sulfation by up-regulation of SULT2A1.[27] Overall CPZ treatment resulted in a relative low number of DEGs in all three cell models, however gene expression changes indicative of an adaptive response in hepatocytes after treatment with CPZ were observed in HepG2 cells and PHH.

Investigation of changes in the drug-induced cholestasis pathway after treatment with EE identified most gene expression changes in HepG2 cells, followed by PMH and PHH, with only one gene expression significantly changed. In PHH, the expression of CYP3A4 was up-regulated after exposure to EE. CYP3A4 is one of the major contributing enzymes involved in the metabolism of EE and thus, up-regulation of this enzyme may be a direct response to EE treatment.[28] Four genes were differentially expressed after EE treatment in PMH, namely BAAT, ABCB11, ABCC3 and ABCB1. BAAT is involved in the conjugation of bile salts and ABCB11, ABAB1 and ABCC3 are involved in the secretion of bile salts. Up-regulation of these enzymes may therefore be part of the adaptive response to EE-induced bile salt accumulation. Genes involved in the export of bile salts were also differentially up-regulated in HepG2 cells, namely OST-β, ABCC2, ABCC3, ABCC4 and ABCB1, indicative of an adaptive response. However, OST-α was down-regulated and the sulfation of bile salts via SULT2A1 was down-regulated. EE was shown to down-regulate mRNA levels of OST-α in the ileum of rats *in vivo*[29] and SULT2A1 is involved in the sulfation of EE.[30] The down-regulation of OST-α and SULT2A1 is not in line with an adaptive response to EE-induced bile salt accumulation. However, down-regulation of these genes may be EE-specific and may play a role in the induction of EE-induced cholestasis.

Overall, investigation of gene expression changes induced by prototypical cholestatic drugs in HepG2 cells, PMH and PHH showed little overlap with gene expression profiles of cholestasis in patients, the HepG2 model performing best. However, investigation of drug-induced expression levels of genes involved in the a priori defined cholestasis pathway revealed some indications for an adaptive response *in vivo* as well as in the *in vitro* liver cell models. Considerable differences in induced gene expressions were observed between compounds and between different cell models. In the present study, only one time point and one concentration were investigated. Extensive dose-dependency studies and time-series analyses may further improve our understanding of drug-induced cholestasis. Furthermore, the development and improvement of new *in vitro* models, e.g. three dimensional cell cultures and the simultaneous culturing of multiple cell types, may better resemble the *in vivo* liver and improve the investigation of drug-induced liver injury.

Table 1. *Numbers of differentially expressed genes (moderate t-test with p-value < 0.05 and absolute FC > 1.5) in in vivo cholestasis and in three in vitro models (HepG2 cells, primary mouse hepatocytes and primary human hepatocytes) after treatment for 24 hours with three cholestasis-inducing compounds (cyclosporin A, chlorpromazine and ethinyl estradiol).*

	Human	HepG2 Cells			PMH			PHH		
	In Vivo	*CsA*	*CPZ*	*EE*	*CsA*	*CPZ*	*EE*	*CsA*	*CPZ*	*EE*
Up-regulated DEGs	1245	1216	201	776	1756	97	362	325	59	8
Down-regulated DEGs	1097	2017	327	1053	1708	173	174	452	70	13

Table 2. *Differentially expressed genes both in in vivo cholestasis samples and in HepG2 cells exposed to cyclosporin A, chlorpromazine and ethinyl estradiol.*

Entrez Gene ID	Gene Symbol	Gene description	GO term
Up-regulated genes			
440	ASNS	Asparagine synthetase (glutamine-hydrolyzing)	Response to amino acid
586	BCAT1	Branched chain amino-acid transaminase 1, cytosolic	Cell proliferation
4131	MAP1B	Microtubule-associated protein 1B	Negative regulation of intracellular transport
6286	S100P	S100 calcium binding protein P	Calcium-dependent protein binding
6809	STX3	Syntaxin 3	Intracellular protein transport; Vesicle-mediated transport
10797	MTHFD2	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	Folic acid-containing compound biosynthetic process
23433	RHOQ	Ras homolog family member Q	Negative regulation of establishment of protein localization to plasma membrane
23643	LY96	Lymphocyte antigen 96	Cell surface receptor signaling pathway; Endosome membrane
55062	WIPI1	WD repeat domain, phosphoinositide interacting 1	Endoplasmic reticulum unfolded protein response
57016	AKR1B10	Aldo-keto reductase family 1, member B10 (aldose reductase)	Steroid metabolic process; Lysosome
57761	TRIB3	Tribbles pseudokinase 3	Negative regulation of fatty acid biosynthetic process; Positive regulation of ubiquitin-protein ligase activity; Response to endoplasmic reticulum stress
84662	GLIS2	GLIS family zinc finger 2	Negative regulation of transcription, DNA-templated; Cell differentiation
Down-regulated genes			
79622	SNRNP25	Small nuclear ribonucleo-protein 25kDa (U11/U12)	mRNA processing

Table 3. *Differentially expressed genes (FC > 1,5; FDR < 0,05 or p-value < 0,05) of the drug-induced cholestasis pathway published by Yuryev et al. in in vivo cholestasis and after in vitro treatment of HepG2 cells, primary mouse hepatocytes and primary human hepatocytes for 24 hours with cholestatic compounds.*

			HepG2 cells			Primary Mouse Hepatocytes			Primary Human Hepatocytes		
Entrez Gene ID	Symbol	In Vivo	CsA	CPZ	EE	CsA	CPZ	EE	CsA	CPZ	EE
Uptake											
10599	SLCO1B1	-1,9**				nh	nh	nh			
6554	SLC10A1		nm	nm	nm	-32,32**				-1,77**	
Synthesis											
1593	CYP27A1					-2,9**					
1581	CYP7A1	-18,77**							nm	nm	nm
Conjugation											
570	BAAT	-1.9*				-21,68**		9.84*			
6822	SULT2A1		-1,66**	2,42**	-2,86**	nm	nm	nm	2,24**		
1576	CYP3A4	-1.63*	nm	nm	nm	nh	nh	nh		2,26**	1,85**
Secretion											
200931	OSTalpha	2,79**	-2,49**		-3,03**	nm	nm	nm	-2,16**		
123264	OSTBETA	10,57**			2,31**	nm	nm	nm			
10257	ABCC4	nm			1,66**						
8647	ABCB11	1.73*	nm	nm	nm	-4,94**		3.03*			
1244	ABCC2		1,59**		2,09**						
8714	ABCC3		2,05**		1,79**	-2,67**		1,68**			
5243	ABCB1		1,53**		2,44**	-2,21**		2,07**			
Nuclear receptors											
9971	NR1H4		1,57**	1.58*		-8,17**					
7421	VDR					nm	nm	nm			
8856	NR1I2	-1.52*				-8,11**					
9970	NR1I3	-1.56*	nm	nm	nm	nm	nm	nm			

* p-value < 0.05 (FDR > 0.05); ** FDR < 0.05; *nm*, Not measured; *nh*, No homologue

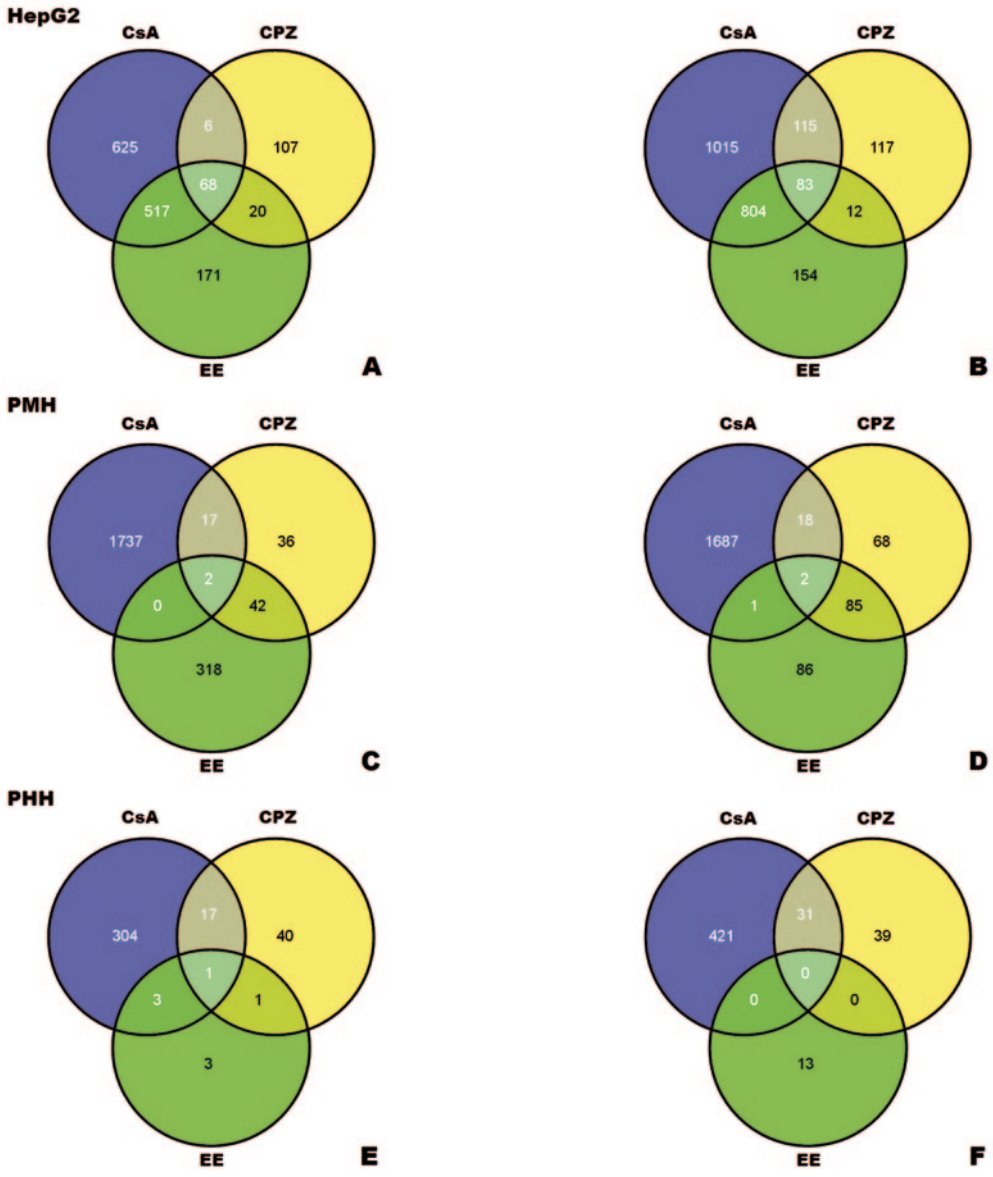

Figure 1. *Venn diagrams showing the overlap in up-, and down-regulated differentially expressed genes (moderate t-test with p-value < 0.05 and absolute FC > 1.5) after treatment with cyclosporin A, chlorpromazine and ethinyl estradiol in HepG2 cells (A and B), primary mouse hepatocytes (C and D) and primary human hepatocytes (E and F).*

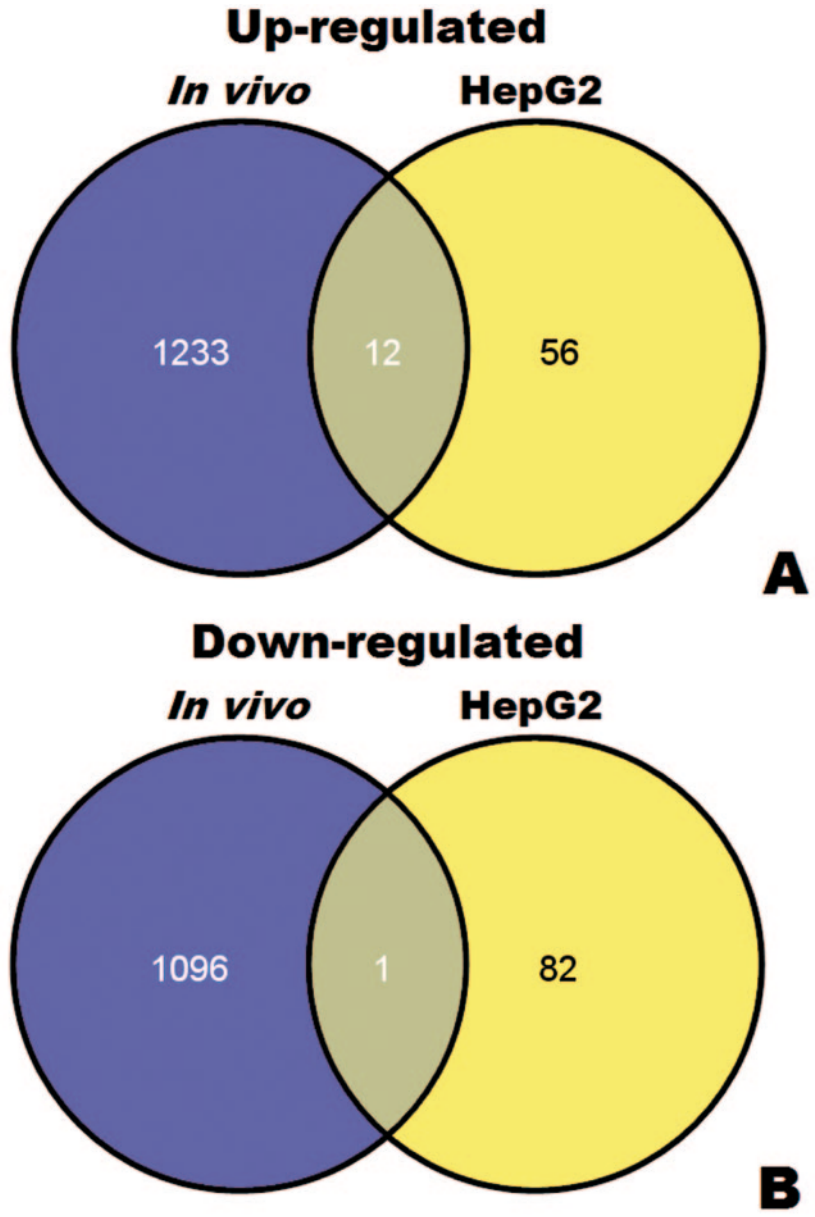

Figure 2. *Venn diagrams showing the overlap in up-regulated (A) and down-regulated (B) differentially expressed genes (moderate t-test with p-value < 0.05 and absolute FC > 1.5) in in vivo cholestasis and in HepG2 cells after treatment with cyclosporin A, chlorpromazine and ethinyl estradiol.*

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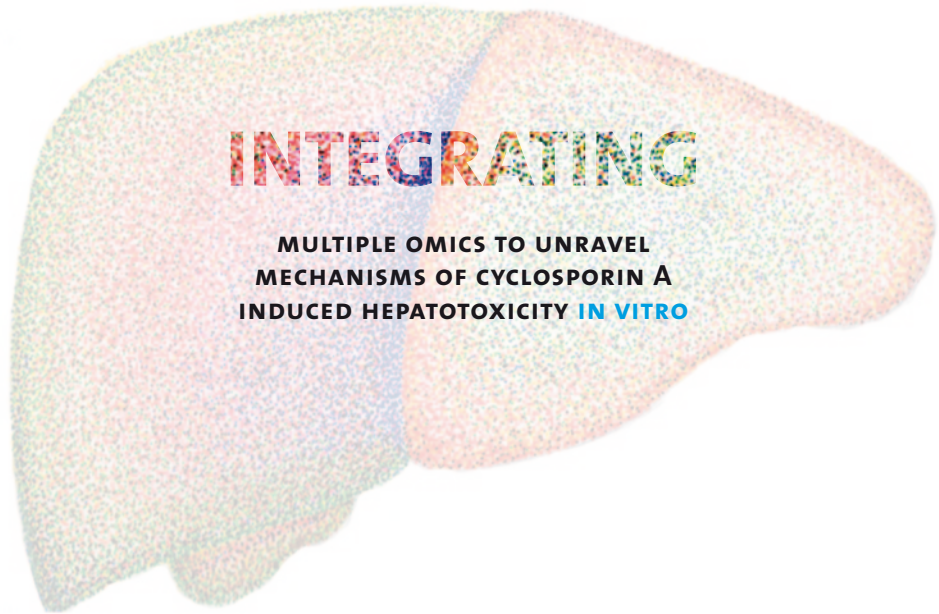
Chapter 4



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Toxicology In Vitro, Revision under review.

Abstract

In order to improve attrition rates of candidate-drugs there is a need for a better understanding of the mechanisms underlying drug-induced hepatotoxicity. We aim to further unravel the toxicological response of hepatocytes to a prototypical cholestatic compound by integrating transcriptomic and metabonomic profiling of HepG2 cells exposed to Cyclosporin A. Cyclosporin A exposure induced intracellular cholesterol accumulation and diminished intracellular bile acid levels. Performing pathway analyses of significant mRNAs and metabolites separately and integrated, resulted in more relevant pathways for the latter. Integrated analyses showed pathways involved in cell cycle and cellular metabolism to be significantly changed. Moreover, pathways involved in protein processing of the endoplasmic reticulum, bile acid biosynthesis and cholesterol metabolism were significantly affected. Our findings indicate that an integrated approach combining metabonomics and transcriptomics data derived from representative *in vitro* models, with bioinformatics can improve our understanding of the mechanisms of action underlying drug-induced hepatotoxicity. Furthermore, we showed that integrating multiple omics and thereby analyzing genes, microRNAs and metabolites of the opposed model for drug-induced cholestasis can give valuable information about mechanisms of drug-induced cholestasis *in vitro* and therefore could be used in toxicity screening of new drug candidates at an early stage of drug discovery.

Introduction

New drugs are being developed continuously. Before they can be released for use by the general public their safety needs to be tested extensively in animal studies and clinical trials. The liver metabolizes many drugs which makes it a vulnerable target for drug-induced toxicity. Despite all the preclinical tests, many drugs are found to be hepatotoxic in phase I to III clinical trials or later [1], indicating that the preclinical tests are not sensitive enough to correctly predict hepatotoxicity.

Hepatotoxicity is one of the most important causes of drug failure, accountable for up to 29% of all drug withdrawals. [2] Drug-induced hepatotoxicity can roughly be divided into three major pathological classes, necrosis, steatosis and cholestasis, and the modes-of-action underlying these pathologies have been investigated extensively. Although multiple mechanisms are identified, some toxicological responses still remain to be clarified. Before clinical testing, drugs are tested in animal toxicity studies, but these studies are time-consuming and are subject to an ongoing ethical debate. Although animal toxicity studies can give valuable information about human toxicity [3], the predictability of animal tests for human toxicity should be improved. [4] Therefore, a major effort is put in finding new, and better, non-animal-based *in vitro* tests for toxicity screening and investigation of toxicological mechanisms.

Toxicogenomics combines toxicology with omics technologies to investigate the mechanisms underlying a toxicological response. [5] Microarray-based gene expression profiling still remains the core technological platform in toxicogenomic research. [6] It is a well-established technique and provides genome-wide information on transcriptomic changes [7] and is used to obtain better insight in the molecular mechanisms underlying drug-induced liver toxicity. [8-10] During the last decade, toxicogenomics research has expanded and also includes transcriptomics of microRNAs, proteomics, metabonomics and epigenomics. MicroRNAs are regulators of mRNA and protein levels and therefore may play an important role in the mechanisms of toxicity. [11] Protein levels as well as post-translational modifications can be measured by proteomic technologies and therewith give valuable information about the mode of action. [12] Metabonomics is defined as 'the quantitative measurement of the time-related multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification' [13] and provides information on the dynamic metabolic status after exposure to a toxicant.

All these 'omics'-technologies individually can generate valuable information about the mechanism underlying drug-induced liver injury. [14-16] It has been shown that integrating transcriptomics with metabonomics data, gives new insights in the molecular mechanisms underlying drug-induced hepatotoxicity *in vivo*. [17-18] Furthermore, Jennen et al. showed that an integrated analysis of transcriptomics and metabonomics data in an *in vitro* experiment with 2,3,7,8-Tetrachlorodibenzo-p-dioxin also resulted in novel insights into response pathways. [19] Although their study also integrated transcriptomics and metabonomics, microRNA profiling was not included. We therefore hypothesize that an integrated transcriptomics and metabonomics approach combined with microRNA profiling will result in an improved understanding of the mechanisms underlying drug-induced hepatotoxicity.

To test this hypothesis HepG2 cells were exposed to the hepatotoxicant Cyclosporin A (CsA). CsA is a calcineurin inhibitor and is widely used as an immunosuppressant in transplant patients. A toxic side effect of CsA is that it could cause cholestasis in transplant patients. [20] This has been confirmed in animal experiments. [21] Additionally, CsA inhibits several ATP-dependent export carriers, including the Bile Salt Export Pump (BSEP, ABCB11). [22-23] The BSEP is responsible for the secretion of bile into the bile canaliculus and inhibition of these transport proteins by CsA will slow down or block the flow of bile, leading to either an intra-hepatocellular build-up of bile-acids or to an increase of serum bile acid levels. Eventually, this will cause hepatocellular damage and induce cholestasis. [24] We consequently decided to exploit this a priori knowledge on CsA-induced hepatotoxicity for evaluating the added value of our integrated omics approach.

Primary human hepatocytes (PHH) are considered to represent the best *in vitro* cell system for liver studies [25], although their use in toxicity screening is bound to limitations. Low availability of fresh human liver samples, difficult culturing procedures and interindividual differences between liver donors hinder the use of PHH for large screening purposes. [26] Therefore, immortalized liver-derived cell lines are thought to present an acceptable alternative and have consequently been used in many toxicity studies. One of these alternatives is the human hepatocellular carcinoma cell line HepG2. Although HepG2 cells have a lower metabolizing capacity compared to PHH [27], several studies demonstrate that HepG2 cells are able to metabolize drugs leading to toxic effects. [28-31] Therefore, HepG2 cells are still frequently used for toxicogenomics and high-throughput toxicity screening studies. [32-40]

HepG2 cells were exposed to different concentrations of CsA for several treatment periods. Bile acid analysis and investigation of the apolar cell extracts was performed for the phenotypic anchoring of cholestatic events. Individual and integrated pathway analyses of transcriptomics and metabonomics data were compared. Mechanisms of CsA-induced hepatotoxicity were further unraveled by investigating the significantly affected pathways and the possible impact of deregulated microRNAs on these pathways.

Materials and methods

Chemicals

Modified Eagle's medium (MEM) plus glutamax, sodium pyruvate, fetal calf serum (FCS), non-essential amino acids, penicillin/streptomycin, Hanks' calcium- and magnesium-free buffer were obtained from Invitrogen (Breda, The Netherlands), dimethylsulfoxide (DMSO), Trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Cyclosporin A (BioChemika), Cholic Acid, Chenodeoxycholic Acid, Deoxycholic Acid, Dehydrocholic Acid and N,N-dimethylformamide (anhydrous, 99.8%) were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands), deuterated chloroform ($CDCl_3$), deuterium oxide (D_2O) and deuterated methanol (CD_3OD) were obtained from VWR International (Amsterdam, The Netherlands) and Ammonium acetate (NH_4Ac), sodium chloride (NaCl), dipotassium hydrogenphosphate (K_2HPO_4), monopotassium hydrogenphosphate (KH_2PO_4) were obtained from Merck (Darmstadt, Germany).

Cell culture

HepG2 cells were cultured in MEM plus glutamax containing 10% v/v FCS, 1% v/v Sodium Pyruvate, 1% v/v non-essential amino acids, 2% w/v penicillin and streptomycin at 37°C in an atmosphere containing 5% CO_2.

Cell treatment

CsA concentrations were derived from cytotoxicity tests based on the MTT method as described in an earlier study. [41] In short, HepG2 cells were treated with 3 µM or 20 µM, which are the IC_{20} concentrations for 72 and 24 hours, respectively. While MTT assays were performed using 2% FCS, different culture conditions requiring different FCS concentrations had to be used in the experiments. 10% FCS was used in the experiments described in this paper, which induced approximately 8% less cytotoxicity (Supplemental file 40). For transcriptomics (mRNA and microRNA) HepG2 cells were seeded in 6 well plates and were exposed for 12, 24, 48 and 72 hours. For metabonomics HepG2 cells were seeded in T75 cm^2 flasks and exposed for 24 and 72 hours. CsA was dissolved in DMSO and added to the medium with a final concentration of 0.5% v/v DMSO. Cells incubated in the presence of 0.5% v/v DMSO served as control. These experiments were performed with three replicates for transcriptomics and five replicates for metabonomics. Replicates were from independent cultures, with passage numbers between 8 and 14.

Phenotypic analyses

Extraction of the apolar metabolites from exposed cells and analysis with 1H-NMR was executed as extensively described by Ruiz Aracama et al. [42] 1H-NMR analysis of apolar extracts were used to investigate fatty acid and cholesterol levels in HepG2 cells, with and without exposure to CsA. Identification of peaks from the apolar extracts was done by using a combination of commercial standards, literature and databases like the Human Metabolome Database. [43]

The bile acids cholic acid, chenodeoxycholic acid, deoxycholic acid and dehydrocholic acid were measured using LC-MS. LC-MS analysis was carried out using a 6410 Triple Quad LC/MS (Agilent Technologies, Santa Clara, USA), equipped with a Agilent 1100 HPLC system with auto sampler and column-oven equipped with an ESI source in negative mode. 10 µL of the cellular extract was injected onto a C18 column (Alltima HP C18-HL 3 µm particle size, 2.1 mm × 150 mm equipped with an Alltima HP guard column C18-HL 5 µm, 2.1 mm × 7.5 mm, Grace Discovery Sciences, Deerfield, USA). Compounds were separated at a flow rate of 200 µL/min using an acetonitrile gradient. Mobile phase A (A) was Acetonitrile + 0.1% formic acid, mobile phase B (B) was demineralized water + 0.1 formic acid. The gradient started at A–B, 5:95, v/v for 4 minutes and increased to 20:80 in 4 minutes and further increased to 95:5 in 5 minutes, which was kept for 3 minutes. The column was then washed with 5:95 for 2 minutes. The column was stabilized at the initial gradient for 13 minutes before injecting the next sample. The total separation time was 27 minutes including stabilization of the column, this was sufficient to achieve baseline separation of several bile acids. The column temperature was maintained at 23°C. After separation, eluting compounds were ionized in ESI in the negative ion mode and detected in full scan. Operating conditions of the MS were optimized using the initial setting as followed: spray voltage of 4 kV, and the heated capillary temperature of 300°C. Nitrogen was used for the sheath and auxiliary gas, set at 10 L/min and 15 psi respectively. The data was processed using the Masshunter Workstation software (Agilent technologies).

Transcriptomics

mRNA expression profiling

Total RNA isolation, target preparation and microarray hybridization on the Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays and scanning on an Affymetrix GeneArray scanner, were performed according to standard procedures. Quality control was performed using the Arrayanalysis.org web service and all microarrays were of high quality. A total of 24 CEL files were obtained, which were re-annotated to EntrezGene IDs using the MBNI BrainArray Custom CDF-files v14 [44], RMA normalized [45] and the intensities were log2 transformed. Correction for False Discovery Rate (FDR) resulted in no significant changes in the low concentration treatments. In order to select biologically relevant changes in all treatments and use identical procedures for all omics analyses, different cutoffs were applied. It was previously reported that combining fold change ranking with a nonstringent p-value cutoff was successful in identifying reproducible gene lists. [7] Therefore, differentially expressed genes (DEGs) for each condition were selected using the following criteria: I) log2 value >6 for at least two replicates, II) for the three replicates an average absolute fold change (FC) of 1.5 (i.e. average log2 ratio of < -0.58 or >0.58), III) same direction of the log2 ratio for all three replicates and IV) a p value <0.05 determined by a paired Student's T-test.

microRNA expression profiling

MicroRNA expression profiling was performed using sixth generation miRCURY LNA microRNA arrays (Exiqon, Vedbaek, Denmark). Reporters on the microarray cover all mature human (1.223), mouse (1.055), rat (680) and related viral (human 146, mouse 57) microRNA sequences as annotated in miRBase v16.0. Each microRNA reporter was present four times on the array. The hybridization was performed following standard protocols. After hybridization, the microarray slides were washed and scanned with a DNA microarray scanner (Agilent Technologies). Feature Extraction Software v10.7.3.1 from Agilent Technologies was used to convert the scanned images into TXT files, which were imported in R 2.14.1 (http://www.r-project.org) for quality control and further downstream analysis. In a first filter step only the viral and human microRNA reporter intensities were retained. Next, these intensities were log2-transformed and quantile normalized. In a final step, the technical replicates of each microRNA were averaged into a single value per microRNA. During this summarization step several criteria had to be met: I) at least 3 out of 4 reporters needed to have a measurable intensity ([foreground] > 1.5x [background]); II) The minimum background-corrected intensity per reporter had to be 10 (unlogged); III) the mean/median ratio of the replicates should not deviate more than 10%. The selection of differentially expressed microRNAs was identical to the last three criteria for mRNAs.

The transcriptomics data discussed in this publication have been deposited in NCBI's gene expression omnibus [46] and are accessible through GEO series accession number GSE45802.

Metabonomics

Extraction of the polar metabolites from exposed cells and analysis with ^{1}H-NMR was done as described by Ruiz-Aracama et al. [42] The NMR data were pre-processed and aligned using an in-house developed program. [47] The aligned fingerprint data in the form of generated spreadsheets were normalized using Probalistic Quotient Normalization [48] combined with log2 transformation. Metabolites were considered to be differentially expressed when the student t test p-value was <0.05 and the absolute FC was >1.5 for all five biological replicates. Polar metabolites were identified by using a combination of commercial standards, literature and databases like the Human Metabolome Database. [43] The results were further used for pathway analyses. Raw data are accessible through Additional file 38.

Pathway analysis

Integrated pathway analyses of transcriptomics and metabonomics data

Lists of differentially expressed genes and metabolites were uploaded into the web-tool 'IMPaLA' [49] for pathway over-representation analysis. Analyses were performed for data generated by the various 'omics' platforms individually as well as combined. Pathways were considered significantly affected if the False Discovery Rate (FDR) was <0.05 and pathways with less than three genes or metabolites were filtered out. Venn diagrams created with VENNY [50] were used to investigate the overlap between the different conditions and Pearson/Ward hierarchical clusters were generated within GenePattern. [51]

Pathway visualization

PathVisio 2.0.11 [52] was used for integrated visualization of the differentially expressed genes, metabolites and microRNAs. Gene database release date 2011-06-01 and metabolite database release date 2011-12-03 was used. The KEGG converted pathway 'Primary bile acid biosynthesis – Homo sapiens (human)' (hsa00120; 2011-05-18) was downloaded from PathVisio and microRNAs were added (see next paragraph). The 'Drug-induced cholestasis pathway' from Ariadne Genomics [53] was adapted by us and microRNAs were included.

Selection of highly conserved microRNA/mRNA targets

For the integration of microRNAs and their mRNA targets custom Perl scripts were used to combine two flat-text files derived from miRanda [54] (release data: Aug 2010) and TargetScan [55] Human (release 60). For miRanda the human 'good mirSVR score, Conserved miRNA' text file was used. For TargetScan this was the 'Predicted Conserved Targets Info' file. As such, both text files only contained highly conserved microRNA targets. The content of both databases was merged based on the stable MIMAT identifier and can be downloaded at http://web.tgx.unimaas.nl/svn/public/miRNA/.

Results

Phenotypic analyses

The focus in bile acids and cholesterol analyses was on four different bile acid standards, namely Cholic Acid, Chenodeoxycholic Acid, Deoxycholic Acid and Dehydrocholic acid, but only Chenodeoxycholic Acid could be detected in intracellular extracts. Chenodeoxycholic Acid was present in the solvent controls with a concentration of 36.50 µg/L (± 4.95), but absent after treatment with 20 µM CsA for 24 hours (Additional file 1) when Chenodeoxycholic Acid levels were diminished and could no longer be detected intracellular.

Intracellular cholesterol levels were measured using ^{1}H-NMR analysis of the apolar extracts. Three different cholesterol pools were identified, namely Esterified Cholesterol, Free Cholesterol and Total Cholesterol. Esterified Cholesterol was significantly lowered after treatment with 3 µM CsA for 72 hours and 20 µM CsA for 24 and 72 hours. The FCs were -1.36, -1.65 and -2.01 respectively. Free Cholesterol and Total Cholesterol increased after treatment with 20 µM CsA for 24 and 72 hours. After 24 hours fold changes were 1.42 and 1.25 and after 72 hours 1.34 and 1.21 for Free Cholesterol and Total Cholesterol respectively.

Cholestasis is defined as a condition in which substances normally excreted into bile, like bile acids, bilirubin and cholesterol, are retained. [56] Accumulation of bile acids due to impaired secretion by hepatocytes or obstruction of bile ducts is the most common condition, but cholesterol accumulation can also be defined as cholestasis. In these experiments cholesterol accumulation was detected and as such is an indication of CsA-induced cholestasis in HepG2 cells.

Transcriptomics and metabonomics

Total numbers of differentially expressed mRNAs, microRNAs and metabolites are summarized in Table 1 and lists are available in the additional files (Additional files 2-20). There is a pronounced dose-related effect, at all time-points more transcripts and metabolites are changed at 20 µM CsA compared to 3 µM. Although less pronounced as the dose-related effect, also a time-dependent effect is observed. Incubating the HepG2 cells with 20 µM of CsA for 72 hours increases the number of differentially expressed mRNAs and microRNAs compared to 12 hours of incubation. At the other time points and for microRNAs and metabolites with the lower concentration the same time-dependent effect is visible.

Venn diagrams showing the overlap of significant genes, microRNAs and metabolites over time and concentration can be found in the additional files (Additional file 21-23). When incubated with 3 µM CsA there were no significant genes overlapping in all time-points, but 737 genes were significantly expressed at all time-points after exposure to 20 µM. Combining all time-points resulted in a total of 150 unique genes differentially expressed during 3 µM CsA exposure of which 137 genes were also differentially expressed during 20 µM CsA treatment. In total 18 unique microRNAs were significantly changed when combining all time-points after 3 µM CsA exposure; seven of these were also found after 20 µM CsA treatment. In total 91 unique microRNAs were significantly affected when combining all time-points after 20 µM CsA exposure. No microRNAs were found which were significantly affected at all time-points

and all concentrations. Combining both time-points and both concentrations, a total of 26 unique metabolites were differentially expressed of which two metabolites were found in all treatments.

Pathway analysis

For functional analysis of CsA-induced omics changes, pathway analyses were performed by using IMPaLA. [49] The microRNA data was excluded from this analysis due to the unavailability to visualize on current pathways. IMPaLA allows an integrated enrichment analysis based on mRNAs and metabolites. Performing integrated pathway analyses on mRNAs and metabolites appears to retrieve more pathways in comparison to single platform-based results (Figure 1).

Integrated analysis of significant mRNAs and metabolites of HepG2 cells exposed to 3 µM CsA for 24 hours resulted in three pathways, which were also found to be significant after pathway analysis of the mRNA data (Figure 1A and Additional file 24). All three pathways play a role in protein processing in the endoplasmic reticulum (ER). Integrated analysis of the data for 20 µM CsA for 24 hours resulted in 169 pathways (Figure 1B and Additional file 25). All pathways which were found to be significant in the pathway analysis of mRNA and metabolite data separately are also found with the integrated analysis, which included pathways involved in cell cycle, cellular metabolism, amino acid metabolism and DNA damage repair. Furthermore, pathways involved in bile, cholesterol and lipid metabolism and pathways involved in detoxification and transport were significantly changed. The integrated analysis resulted in three extra significant pathways which are all involved in the amino acid metabolism.

Integrated analysis of the results for cells exposed to 3 µM CsA for 72 hours resulted in one significant pathway, purine metabolism, which was also found to be significant in the pathway analysis of the metabolite data (Figure 1C and Additional file 26). Integrated pathway analysis for the 20 µM CsA data resulted in 231 significant pathways of which 64 were not found with the separate omics analyses (Figure 1D and Additional file 27). Six pathways were found under all conditions of which five were involved in the amino acid metabolism and one was involved in cellular metabolism. 48 pathways were overlapping in the analyses of the mRNA data and integrated data and 77 pathways overlapped between metabonomic and integrated data. These pathways were involved in cell cycle and cellular metabolism like energy and amino acid metabolism and processes like cholesterol and (trans-membrane) transport of molecules were found. Most of the pathways that were only found with the integrated analysis were involved in cellular mechanisms also found with the separate analyses, like cell cycle, cholesterol and lipid metabolism and pathways involved in detoxification, transport and protein processing of the ER were significantly enriched. However, integrated analysis showed multiple pathways involved in glucose and bile homeostasis, where only 1 or 2 of these pathways were found with the separate analyses.

Integrated pathway analysis of transcriptomic and metabonomic data results in more significantly affected pathways and included all pathways which are found with the pathway analyses of the separate omics. Furthermore, the extra pathways found by integrating multiple omics give more information about the cellular response to drug-induced toxicity.

Pattern analysis of significant pathways

In order to gain insight in the time- and dose-related patterns of the affected pathways and possible associations between or grouping of pathways, a hierarchical clustering analysis was performed. Pathways which were significant in at least one of the treatments were selected and hierarchical cluster analysis was performed using the –log p-values (Figure 2). The first cluster consists of five pathways which are mainly affected at 24 hours and 3 µM and are involved in amino acid metabolism and protein processing of the ER (Additional file 28A and 29). The second cluster with main effects at 24 hours and 20µM, mostly included pathways involved in cell cycle, DNA damage repair and amino acid metabolism covering 73% of all significant pathways in this cluster (Additional file 28B and 30). The third cluster with pathways affected after 72 hours and 20 µM mostly included pathways involved in amino acid and energy metabolism covering up to 55% of all pathways in this cluster (Additional file 28C and 31). Moreover, the percentage of pathways involved in cholesterol and bile metabolism and pathways involved in blood clotting and transport were increased when comparing the second and third cluster, where the percentage of pathways involved in cell cycle were decreased.

MicroRNA transcriptomics

In order to further unravel the mechanisms underlying CsA-induced hepatotoxicity, microRNA expression levels were investigated. Since microRNAs can negatively regulate targeted mRNA levels, we explored whether such correlating effects occurred within our data set. For all the differentially expressed microRNAs, the differentially expressed mRNAs were searched for their targets and checked if (anti-)correlating combinations were significantly enriched. Despite that many of those combinations were found, no significant enrichment was observed, meaning that in this study the effects on mRNAs cannot be explained by direct regulation through microRNAs.

In order to gain insight in the time- and dose-related patterns of the differentially expressed microRNAs and possible associations between or grouping of these, a clustering analysis was performed (Figure 3). To understand in which biological pathways, thus possible mechanisms, the microRNAs are involved, for each cluster the predicted target genes were retrieved and enrichment analyses were performed on these target mRNAs in IMPaLA.

The first cluster (Figure 3; cluster 1) consists of only one microRNA, hsa-miR-27a. This microRNA is slightly elevated after exposure to 3 µM and 20 µM CsA for 12 hours, with a FC of 1.06 and 1.20 respectively. Over time the FC increases and after 72 hours exposure to 3 µM and 20 µM CsA a FC of 1.40 and 1.69 is reached, respectively. In total this microRNA has 886 target genes of which 293 genes were differentially expressed within the mRNA experiment, although no significant enrichment of correlation or anti-correlation occurs. Pathway analysis indicates that the target genes of this microRNA are involved in 99 significant pathways (Additional file 32), which are mostly related to cell cycle, immune response and energy metabolism. Furthermore, the significant pathways included the SREBP signaling pathway, an important pathway in the cellular lipid homeostasis.

Thirteen microRNAs were found in the second cluster (Figure 3; cluster 2) which are

increased after 72 hours incubation with CsA regardless of the concentration and most are down regulated after 12, 24 and 48 hours incubation. The microRNAs in this cluster have a total of 4.914 target genes and 1.620 of these genes were significantly affected in this experiment, again without significant enrichment of (anti-)correlation effects. Pathway analysis of all target genes resulted in 478 significant pathways (Additional file 33), which were mostly involved in cell cycle processes. Furthermore, pathways were involved in immune response, calcium homeostasis, lipid metabolism, protein processing and trans-membrane transport.

The third cluster (Figure 3; cluster 3) consists of two microRNAs, hsa-miR-18b and hsa-miR-183. These microRNAs are down regulated at 48 hours 20 µM, but are up regulated or not changed at the other time-points. A total of 514 target genes were found for these two microRNAs and 195 of these target genes were significantly changed. Pathway analysis of all target genes resulted in nine significantly changed pathways (Additional file 34) which were mostly related to cell cycle processes but also pathways involved in development and protein processing were found.

The fourth cluster (Figure 3; cluster 4) consists of five microRNAs of which the FCs were lower at 48 and 72 hours compared to 12 and 24 hours both with 3 µM and 20 µM and most microRNAs were down-regulated at 48 and 72 hours. In total 2196 target genes were found of which 743 were significantly changed in this experiment. 218 pathways were found to be significantly changed (Additional file 35) and these were mostly involved in cell cycle processes. Moreover, significant pathways included pathways involved in immune response, calcium homeostasis and synaptic transmission, in which calcium is highly involved.

The fifth cluster (Figure 3; cluster 5) consists of ten microRNAs which were down regulated at all time-points with 3 µM CsA. When exposed to 20 µM CsA, all microRNAs were down regulated after 12, 48 and 72 hours, but most were up regulated after 24 hours. A total of 2860 target genes were found for this cluster of which 965 were significantly changed in this experiment. 234 pathways were significantly changed when all target genes were used for pathway analysis (Additional file 36) and these were generally involved in cell cycle and developmental processes. Moreover, pathways involved in cancer, apoptosis, immune response and calcium homeostasis were found.

In total 536 unique microRNA-related pathways were found to be significantly affected of which most could be related to cell cycle processes, immune response and calcium homeostasis. Although identified microRNA-mRNA complexes did not demonstrate concordant interactions as expected this finding is in concordance with the hypothesis that integrated analysis of data generates added value.

Visualization of effects in pathways involved in bile acid metabolism

Both the phenotypic analyses and the omics analyses indicate that CsA causes effects on cholesterol and bile metabolism. To visualize the effects of CsA on mRNAs, microRNAs, metabolites, cholesterol and bile acids two pathways involved in bile acid metabolism were retrieved and visualized with PathVisio. The first pathway, bile acid biosynthesis, was found multiple times; for visualization the KEGG converted pathway 'Primary bile acid biosynthesis' was used (Figure 4). Nine of the 17 genes and three of the 47 metabolites, including cholesterol and Chenodeoxycholic acid, were significantly altered in at least one of the experimental conditions. The second pathway was the 'Drug-induced cholestasis pathway' from Ariadne Genomics [53] which was setup as a model for drug-induced cholestasis and was adapted by us to visualize the data (Figure 5). Eleven out of the 18 genes were significantly affected in at least one of the treatments.

Since these pathways lacked any information on microRNAs, we added these based on predicted microRNA-mRNA pairs based on TargetScan and miRanda. Three of the 18 genes in the 'Drug-induced cholestasis pathway' may be regulated by microRNAs and eight of the 17 genes of the 'Primary bile acid biosynthesis'. In total 23 and 46 microRNAs may be involved in the 'Primary bile acid biosynthesis' pathway and the 'Drug-induced cholestasis' pathway, respectively. In both pathways six microRNAs were differentially expressed. The effects of CsA on mRNAs, microRNAs, metabolites, cholesterol and bile acids were visualized in Figure 4 and 5.

The 'Primary bile acid biosynthesis' pathway is overall down-regulated, which is in accordance with the phenotypic data, namely that the input of the pathway, cholesterol, is up-regulated and the output, Chenodeoxycholic acid, is down-regulated.

Most of the genes of the 'Drug-induced cholestasis' pathway were also down regulated, especially the genes involved in the synthesis and conjugation of bile acids which is consistent with the expression of genes in the 'Primary bile acid biosynthesis' pathway. Although no significant enrichment of correlation and anti-correlation of mRNA-microRNA pairs was found, these data suggests that microRNAs may play a role in the bile acid biosynthesis and drug-induced cholestasis.

Discussion

In this study, we aimed to unravel the mechanisms underlying CsA-induced hepatotoxicity by integrating transcriptomics and metabonomics with microRNAs. HepG2 cells were used to study the CsA-induced effects *in vitro*. In order to check endpoints of the CsA-induced toxicity in HepG2 cells, phenotypic analyses of cholesterol and bile acids were performed. We showed an increase of total cholesterol and free cholesterol and a decrease of esterified cholesterol. Intracellular cholesterol accumulation can lead to reduced cholesterol uptake by hepatocytes and increased cholesterol secretion, which corresponds to *in vivo* cholestasis where an increased serum cholesterol level is an important marker for cholestasis. [57] Up to a certain concentration level, free cholesterol is an important part of the cellular membrane, but a rise in free cholesterol levels can change the ratio of free cholesterol and phospholipids and impair the function of membrane proteins. Furthermore, high levels of free cholesterol can induce mitochondrial and ER-stress. [58] Free cholesterol is therefore esterified to be stored or secreted as free and esterified cholesterol via lipoproteins. [59] ACAT2 is the most important enzyme involved in cholesterol esterification in hepatocytes. [60] It is significantly down-regulated after 3 µM CsA exposure for 24 hours with a FC of -1.36 and after 20 µM CsA exposure for 24 and 72 hours with a FC of -2.19 and -4.51, respectively. This may explain the decrease of esterified cholesterol levels.

Moreover, free cholesterol can be broken down towards bile acids and secreted into bile. There

are two primary bile acids formed in human liver, namely cholic acid and chenodeoxycholic acid. Both bile acids can be formed via the classic bile acid biosynthesis pathway of which CYP7A1 is the rate limiting enzyme. Chenodeoxycholic acid can also be formed via the alternative bile acid biosynthesis pathway of which CYP27A1 is the initiating enzyme (Figure 4). [61] No cholic acid was detected in solvent controls or in CsA-treated cells, which suggests that the classic bile acid biosynthesis in these HepG2 cells is low or absent. It has previously been reported that CYP7A1 expression levels are low in HepG2 cells [62], which was confirmed in this study where CYP7A1 log2 expression was below the selection criteria of 6. Low CYP7A1 expression levels could result in low cholesterol 7α-hydroxylation and subsequent processes of the classic bile acid biosynthesis pathway and therefore making the alternative CYP27A1-mediated pathway the main bile acid biosynthesis pathway. Chenodeoxycholic acid levels were detected in solvent controls and were diminished after CsA treatment, which suggests that CsA treatment inhibits the alternative bile acid biosynthesis. Previous studies showed a decreased activity of CYP27A1 after CsA exposure, resulting in a down-regulation of the alternative bile acid biosynthesis pathway [63-64], which was confirmed in our study. CYP27A1 was significantly down-regulated after 20 µM CsA exposure for 48 and 72 hours with a FC of -1.76 and -2.2, respectively.

Transcriptomic and metabonomic responses to CsA exposure were investigated and affected processes were analyzed using integrated pathway analysis. A distinct dose-dependent effect was observed after CsA treatment, total number of significantly expressed mRNAs, microRNAs and metabolites being increased as a result of exposure to a higher CsA concentration. Therapeutic blood levels of CsA in patients can be up to 0.67 µM [65], thus our experimental concentrations were 5-30 fold higher. We choose these higher levels based on a previous study [41], with the aim to induce a distinct toxic effect, with only a minor loss in viability. Incubating HepG2 cells with 3 µM CsA for 72 hours resulted in a loss of 20% viability [41], while incubating the HepG2 cells with 20 µM CsA resulted in a loss of 20% viability after 24 hours and a loss of 50% viability after 72 hours (Additional file 37). The IC_{20} and IC_{50} values are often used as a measure for cytotoxicity in *in vitro* studies, including toxicogenomic studies. Apart from the dose effect, also a distinct time-dependent effect was found, which was clearly seen when the pathway analysis results were analyzed with hierarchical clustering. With respect to the pathways, one small and two large clusters were found which both had a different dose/time pattern. Most of the pathways affected after 24 hours were related to cell cycle processes and DNA damage response, which could suggest an acute stress response of the exposed cells. Furthermore pathways related to amino acid metabolism and protein processing were affected, which can be related to processes of the ER. After 72 hours, pathways involved in amino acid, energy and calcium metabolism were affected in which the ER and mitochondria play an important role, which could be the result of a cytotoxic response to the long incubation with CsA. Furthermore, pathways involved in cholesterol and bile acid metabolism are found to be significantly affected which is in line with the results of the phenotypic analyses. Pathways related to calcium metabolism and ER functions can be related to the disruption of the mitochondrial and ER membranes by the accumulation of free cholesterol [58] and could therefore be a general cholestasis effect. Furthermore, ER stress could be a result of a direct effect of CsA on cyclophilin B. Cyclophilin B is a protein in the ER which is important for proper protein folding [66] and previous studies showed that CsA can impair the function of this protein resulting in an accumulation of misfolded proteins in the ER and ER stress. [67] Furthermore, ER stress and subsequent changes in calcium homeostasis and mitochondrial function have been related to several liver diseases, including cholestasis [68], and CsA-induced hepatotoxicity. [69] Future research, using multiple cholestatic compounds, should asses if the changes induced by CsA are truly specific for cholestasis or are compound specific.

Cytochrome P450 enzymes are the major enzymes involved in drug metabolism and are involved in steroid, cholesterol and bile acid metabolism. Bile acid biosynthesis is an important part of the opposed model for drug-induced cholestasis (Figure 5). Besides down regulation of the transcription of the key enzymes in the classic and alternative pathways also the downstream genes in these pathways were down regulated, especially at the high CsA concentration, as can be seen in Figure 4. Figure 5 shows a proposed model of drug-induced cholestasis in which four processes of bile acid metabolism are incorporated, namely the uptake, synthesis, conjugation and secretion of bile acids. The uptake of bile acids is down-regulated when bile acids accumulate in hepatocytes. [70] However, it has been shown that the functionality of uptake-transporters for bile acids in HepG2 cells is limited. Kullak-Ublick et al. showed that the Na^+-taurocholate co-transporting polypeptide (NTCP/SLC10A1) is not active in HepG2 cells, but there was uptake of taurochate via the organic anion transporting polypeptide (OATP) which had an expression of 40% in comparison with normal liver. [71] In our study the expression level of both SLCO1B1 (OATP1B1) and SLC10A1 (NTCP) did not pass our detection filter and thus deregulation thereof could not be investigated. Bile acid synthesis was significantly down-regulated after CsA treatment. Conjugation of bile acids and xenobiotics is necessary to increase the secretion. BAAT is responsible for conjugation of bile acids with taurine and glycine, but both BAAT and glycine are significantly affected after 20µM CsA treatment for 72 hours. BAAT gene expression is down-regulated with a FC of -3.03 and glycine is up-regulated with a FC of 1.97 suggesting a down-regulation of glycine conjugation of bile acids. Sulfation is also an important pathway for detoxification and secretion of bile acids and is regulated in hepatocytes by SULT2A1 [72], which was significantly down-regulated after 20 µM CsA treatment for 48 and 72 hours with a FC of -3.16 and -6.54, respectively. CYP3A4 is one of the most important enzymes involved in the metabolism of xenobiotics, including CsA, but is only significantly up-regulated after 20 µM CsA treatment for 12 hours with a FC of 1.24. The last important step in this model for drug-induced cholestasis is the secretion from hepatocytes. Inhibition of bile acid secretion via the BSEP has been related to cholestasis and CsA is a known inhibitor. However, expression levels are low in HepG2 cells [73-74] and no effect of CsA was found. Several other proteins that can secrete bile acids and xenobiotics, OSTalpha, OSTbeta, ABCC2, ABCC3, ABCC4 and ABCB1, are expressed [75-76] and functional in HepG2 cells although in lower levels than in human liver. [77] In our study OSTalpha and OSTbeta were down-regulated after 20 µM CsA treatment, where ABCC2, ABCC3, ABCC4 and ABCB1 were up-regulated.

To complete the model, several important nuclear receptors involved in bile acid metabolism [78] are added in Figure 5. The farnesoid X receptor (FXR; NR1H4) is a nuclear receptor for which

Chenodeoxycholic acid and other bile acids are natural ligands, and regulates genes involved in the bile acid biosynthesis. The FXR is significantly down-regulated after 20 µM CsA treatment for 72 hours compared to the solvent control, which could be the result of the diminished levels of Chenodeoxycholic acid after CsA exposure. The pregnane X receptor (PXR; NR1I2) is a nuclear receptor which up-regulates proteins involved in detoxification and is significantly up-regulated after 20 µM CsA treatment for 72 hours. The vitamin D receptor (VDR; NR1I1) and constitutive androstane receptor (CAR; NR1I3) were also shown to be activated by bile acids or bile acid metabolites.

Analysis of microRNAs and mRNAs did not result in significant enrichment of correlating or anti-correlating combinations, although many microRNA-mRNA combinations were found. Comparing the pathway analyses of the target genes of microRNAs with the same time- and dose-related patterns to the integrated pathway analyses of mRNAs and metabolites resulted in pathways involved in similar processes, indicating a possible role for microRNAs in the cellular reaction on CsA exposure. Furthermore, several microRNAs were found to have target genes located in the bile acid biosynthesis and 'drug-induced cholestasis' pathways which were affected in CsA treated HepG2 cells. Involvement of microRNAs in drug-induced toxicity has not been studied extensively, but microRNA expression levels could be important to elucidate all mechanisms underlying a cellular reaction to a compound, because microRNAs are able to post-transcriptionally alter protein expressions. Further research is needed to investigate the reaction of microRNAs to CsA and their ability to regulate the target genes and corresponding protein levels in drug-induced cholestasis.

Conclusions

We could unravel mechanisms of CsA-induced hepatotoxicity using an integrated-omics approach, both by combining transcriptomics and metabonomics and by including microRNAs. Data analysis using the proposed model for drug-induced cholestasis gives valuable information about important genes, microRNAs and metabolites involved in bile acid metabolism in hepatocytes. This model could therefore be used to screen new compounds for their ability to influence bile acid metabolism, which could be an indication of cholestatic properties. However, validation of this model using multiple compounds, including non-cholestatic drugs, should be performed. Several differentially expressed genes were related to ER stress and mitochondrial impairment after CsA exposure. Cytochrome P450 enzymes were also affected which resulted in intracellular cholesterol accumulation due to impairment of cholesterol metabolism and bile acid synthesis. We showed that integrating multiple omics and thereby analyzing genes, microRNAs and metabolites of the opposed model for drug-induced cholestasis can give valuable information about mechanisms of drug-induced cholestasis *in vitro* and therefore could be used as addition in toxicity screening of new drug candidates at an early stage of drug discovery. Further research is needed to validate our results in different models. Furthermore, multiple cholestatic compounds should be used to separate the compound specific changes from the true cholestatic mechanisms.

Availability of supporting data

The data sets supporting the results of this article are included within the article (and its additional files) or are available in the NCBI's gene expression omnibus. The transcriptomics data are accessible through GEO series accession number GSE45802. Additional files are available upon request.

Acknowledgements

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Table 1. *Numbers of differentially expressed mRNAs, microRNAs and metabolites induced in HepG2 cells after cyclosporin A exposure across time and concentration.*

	3 µM				20 µM			
	12h	*24h*	*48h*	*72h*	*12h*	*24h*	*48h*	*72h*
mRNA	**89**	**72**	**20**	**29**	**2189**	**2130**	**3004**	**3095**
Up-regulated	82	61	17	20	1001	838	1431	1494
Down-regulated	7	11	3	9	1188	1292	1573	1601
microRNA	**1**	**0**	**7**	**11**	**8**	**8**	**62**	**43**
Up-regulated	1	0	3	9	8	7	30	30
Down-regulated	0	0	4	2	0	1	32	13
Metabolites	**NA**	**5**	**NA**	**10**	**NA**	**16**	**NA**	**16**
Up-regulated	-	3	-	5	-	8	-	10
Down-regulated	-	2	-	5	-	8	-	6

NA, not analyzed

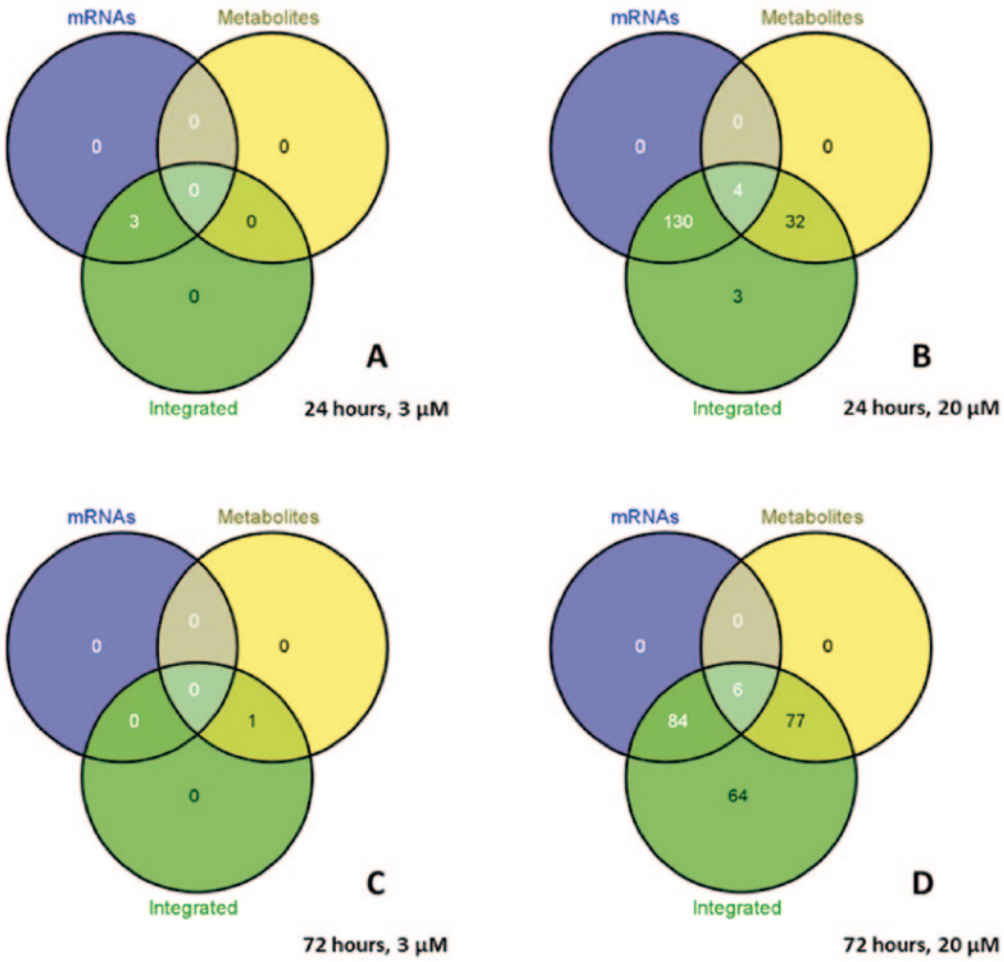

Figure 1. *Venn diagrams showing the overlap of significantly changed pathways (FDR<0.05; IMPaLA) between transcriptomics and metabonomics separately and integrated.*

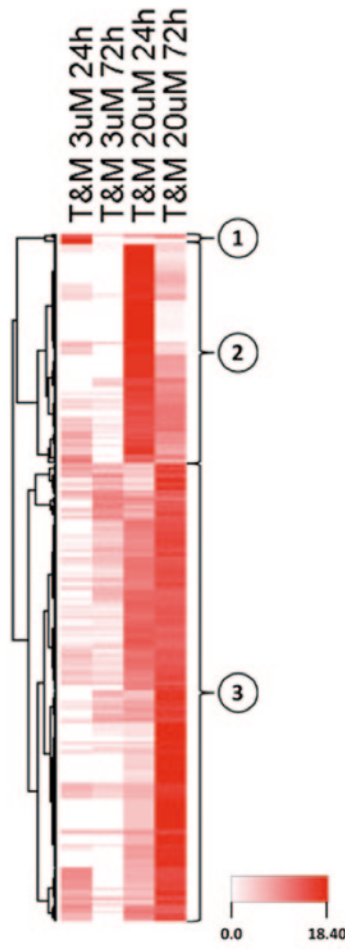

Figure 2. *Hierarchical clustering of pathways which were significantly affected in at least one of the treatments based on their –log p-values.*

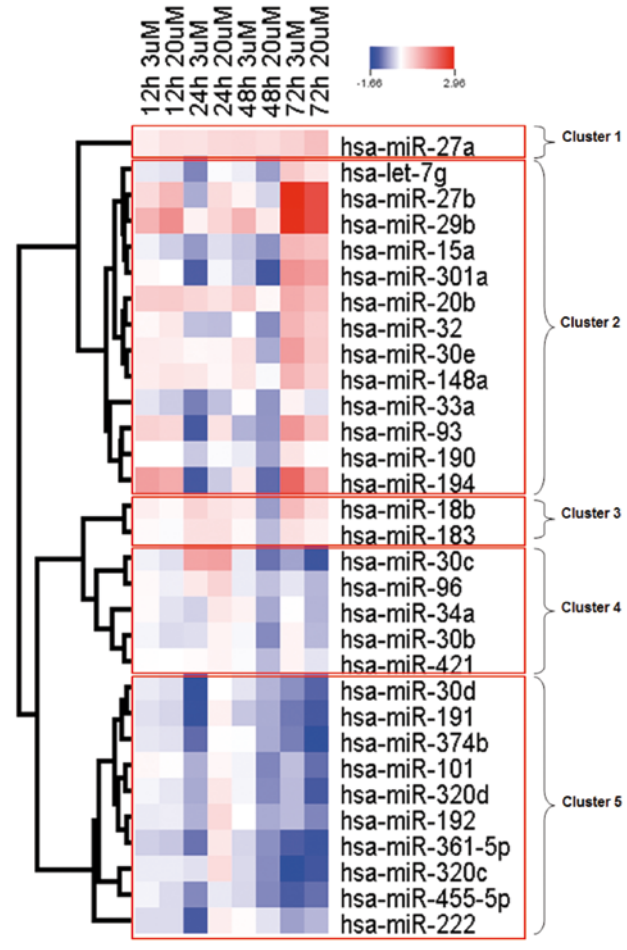

Figure 3. *Hierarchical cluster analysis of microRNAs, which were significant in at least one of the treatments and are known to have target genes, based on the log2 of their FC.*

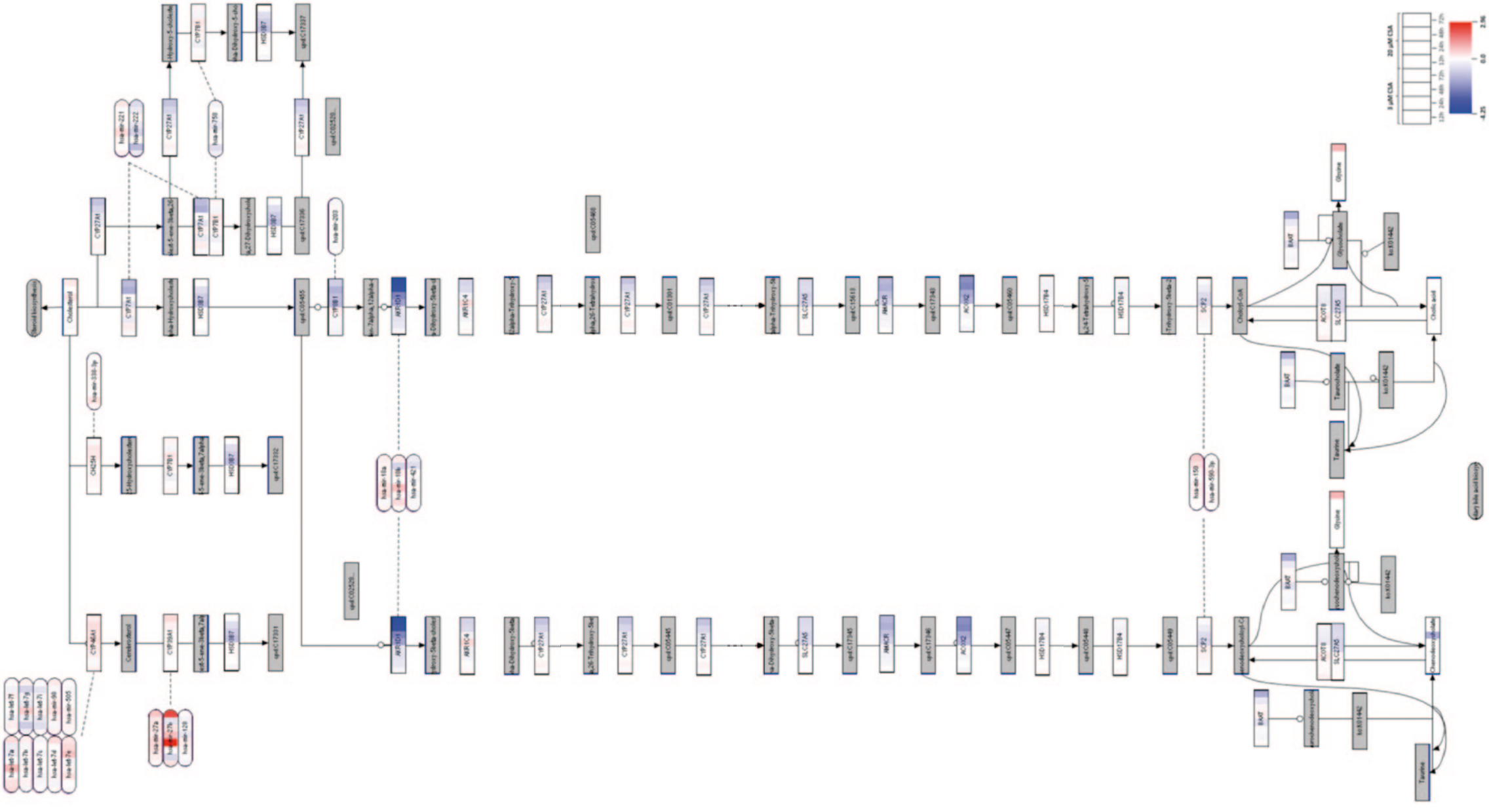

Figure 4. *Visualization of expression levels (log2 FC) of all measured transcriptomics, metabonomics and phenotypic data in the Kegg pathway 'Primary bile acid biosynthesis'.*

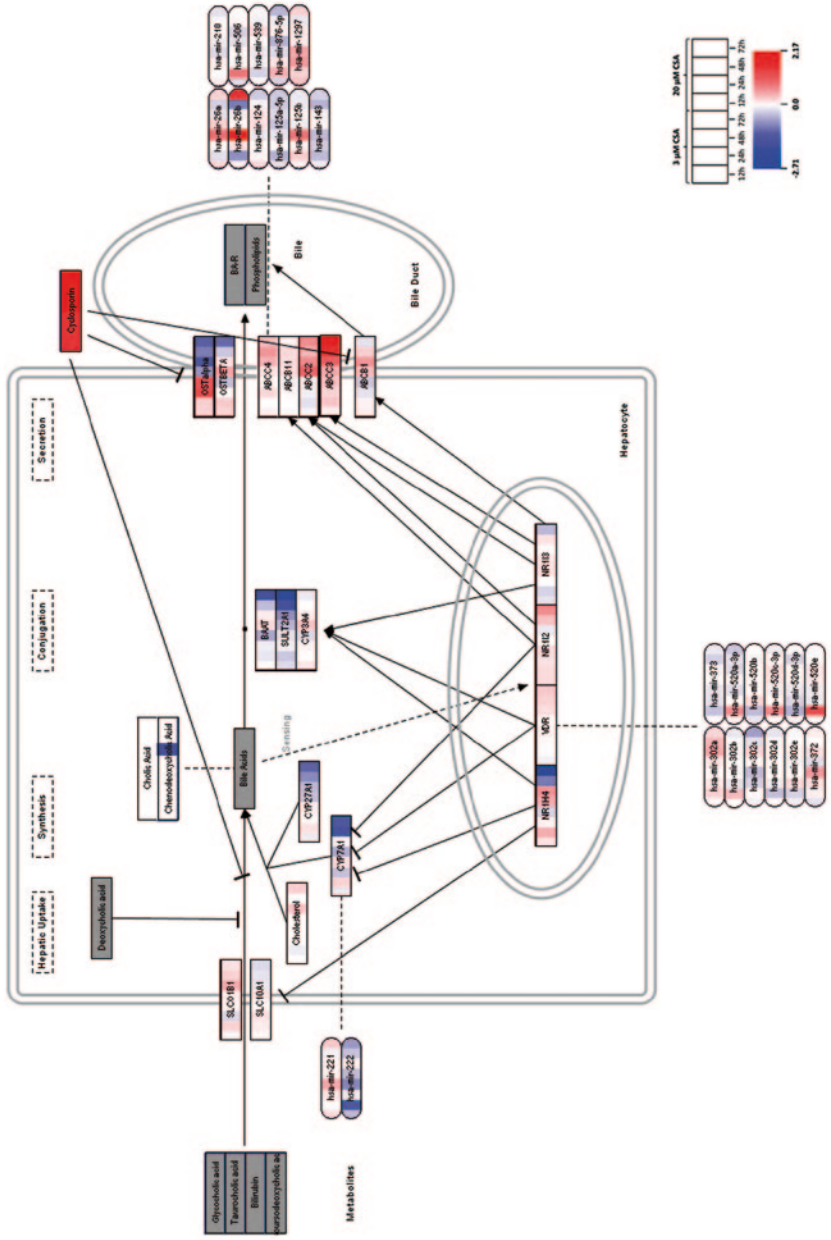

Figure 5. *Visualization of expression levels (log2 FC) of all measured transcriptomics, metabonomics and phenotypic data in the 'Drug-induced cholestasis' pathway, which is suggested to be a model for drug-induced cholestasis.*

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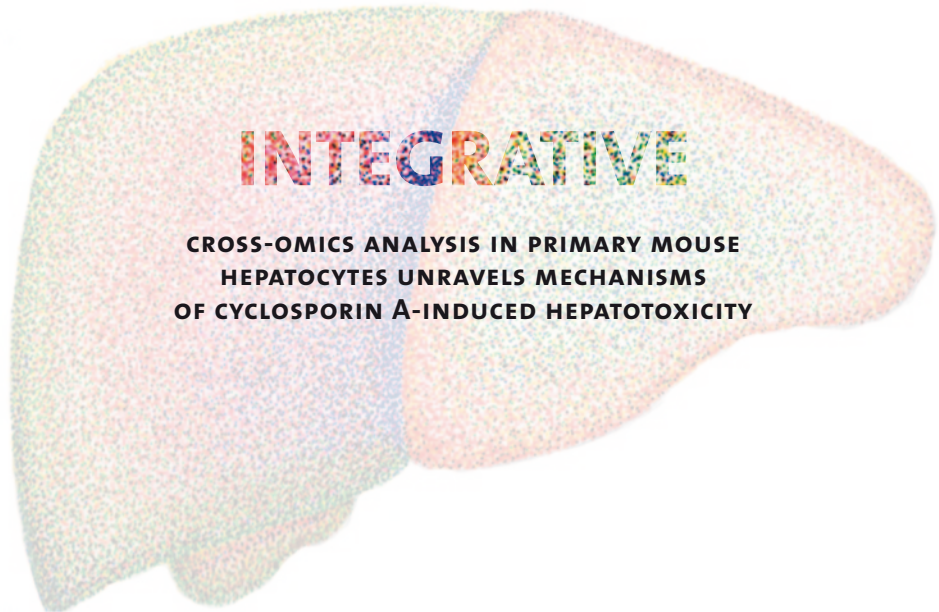
Chapter 5



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Abstract

The liver is responsible for drug metabolism and drug-induced hepatotoxicity is the most frequent reason for drug withdrawal, indicating that better pre-clinical toxicity tests are needed. In order to bypass animal models for toxicity screening, we exposed primary mouse hepatocytes for exploring the prototypical hepatotoxicant cyclosporin A. To elucidate the mechanisms underlying cyclosporin A-induced hepatotoxicity, we analyzed expression levels of proteins, mRNAs, microRNAs and metabolites.

Integrative analysis of transcriptomics and proteomics showed that protein disulfide isomerase family A, member 4 was up-regulated on both the protein level and mRNA level. This protein is involved in protein folding and secretion in the endoplasmic reticulum. Furthermore, the microRNA mmu-miR-182-5p which is predicted to interact with the mRNA of this protein, was also differentially expressed, further emphasizing endoplasmic reticulum stress as important event in drug-induced toxicity. To further investigate the interaction between the significantly expressed proteins, a network was created including genes and microRNAs known to interact with these proteins and this network was used to visualize the experimental data. In total 6 clusters could be distinguished which appeared to be involved in several toxicity related processes, including alteration of protein folding and secretion in the endoplasmic reticulum. Metabonomic analyses resulted in 5 differentially expressed metabolites, indicative of an altered glucose, lipid and cholesterol homeostasis which can be related to cholestasis.

Single and integrative analyses of transcriptomics, proteomics and metabonomics reveal mechanisms underlying cyclosporin A-induced cholestasis demonstrating that endoplasmic reticulum stress and the unfolded protein response are important processes in drug-induced liver toxicity.

Introduction

Rodent studies have been used for decades to predict toxicological properties of compounds and are obligatory before admission of new compounds onto the market. Although these studies provide valuable information on the safety of these compounds, rodent tests do not always prevent that actual human toxicants advance into clinical trials and onto the market. The liver is the most important organ for drug metabolism, making it a vulnerable target for drug-induced toxicity. Drug-induced hepatotoxicity is the most frequent reason for the withdrawal of a drug after it has already been approved. [1] Furthermore, ethical policies require replacement, reduction and refinement of animal tests. One way of reducing the number of rodents used for toxicity screening, is the use of *in vitro* alternative models.

The gold standard for *in vitro* hepatotoxicity screening is based on the application of primary human hepatocytes. [2] However, the use of primary human hepatocytes has some important drawbacks. Hepatocyte donors are scarce, interindividual variation is high and the culture conditions are laborious. By contrast, where most animal-based toxicity screenings are performed with mice, it is suggested that primary mouse hepatocytes present a relevant alternative *in vitro* liver model, even more so because of their easy availability and low interindividual variation. Furthermore, primary mouse hepatocytes cultured in a collagen sandwich configuration, maintain their metabolic competence for up to 90 hours, thus enabling the investigation of drug metabolism and hepatotoxicity over a considerable period and at multiple time-points. [3]

Classical markers, such as alanine transferase and alkaline phosphatase, represent the standard for assessing liver damage. However, specificity is low and an increase is only noticed after cell death has occurred. [4] Therefore, there is a need for specific and sensitive mechanistic markers, in particular for the early detection of hepatotoxicity. Transcriptomics, proteomics and metabonomics are techniques capable of detecting early molecular changes upon exposure to a toxicant and have all been used to investigate the mechanisms underlying drug-induced toxicity. [5-7] Currently, much effort is being put in combining these different techniques into a systems biology approach.

In the present study, we therefore evaluated multi-omics analysis of drug-induced hepatotoxicity using primary mouse hepatocytes. The prototypical human hepatotoxicant cyclosporin A (CsA) was used to induce toxicity in the mouse *in vitro* liver model. CsA is a widely used immunosuppressant drug and capable of inducing cholestasis *in vivo*. Cholestasis occurs when substances normally excreted in the bile, accumulate intracellularly or in the bile ducts. It is suggested that CsA inhibits several export pumps in hepatocytes, like the bile salt export pump (BSEP), multidrug resistance protein 1 (MDR1) and multidrug resistance-associated protein 2 (MRP2). The inhibited export will result in bile acid accumulation in hepatocytes, eventually leading to cell damage and leaking of bile acids into the bloodstream. Recently, Vinken et al. published an Adverse Outcome Pathway (AOP) from BSEP inhibition to cholestatic liver injury caused by the accumulation of bile acids. [8]

By analyzing expression levels of proteins, mRNAs, microRNAs and metabolites, we aim to further elucidate the mechanisms behind CsA-induced hepatotoxicity and to investigate the processes leading to CsA-induced cholestasis.

Materials and methods

Chemicals

Modified Eagle's medium (MEM) plus glutamax, sodium pyruvate, fetal calf serum (FCS), non-essential amino acids, penicillin/streptomycin, Hanks' calcium- and magnesium-free buffer and insulin were obtained from Invitrogen (Breda, The Netherlands). Glucagon, hydrocortisone (water soluble), collagenase type IV, dimethylsulfoxide (DMSO), Trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Cyclosporin A (BioChemika) and N,N-dimethylformamide (anhydrous, 99.8%), NaCl, $NaHCO_3$, KCl, KH_2PO_4, $MgSO_4$, glucose, and $CaCl_2$ were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Collagen type I Rat Tail was obtained from BD Biosciences (Bedford, MA, USA). Deuterated chloroform ($CDCl_3$), deuterium oxide (D_2O) and deuterated methanol (CD_3OD) were obtained from VWR International (Amsterdam, The Netherlands) and Ammonium acetate (NH_4Ac), sodium chloride (NaCl), dipotassium hydrogenphosphate (K_2HPO_4), monopotassium hydrogenphosphate (KH_2PO_4) were obtained from Merck (Darmstadt, Germany). The Trizol reagent and the RNeasy mini kit were from Qiagen Westburg (Leusden, The Netherlands).

Animals

The animal studies were approved by the Animal Ethical Committee of Maastricht University, The Netherlands (approval number: 2011-108). Adult male C57BL/6 mice, weighing 20-25 grams, were obtained from Charles River GmbH, Sulzfeld, Germany. Animals were housed in macrolon cages with sawdust bedding at 22°C and 50-60% humidity. Food and tap water were available *ad libitum*, and the light cycle was 12 hours light/12 hours dark.

Isolation and culturing of primary mouse hepatocytes

A two-step collagenase perfusion method according to Seglen [9], with modifications as described before [3] was used to isolate hepatocytes. In short, cell suspensions with cell viability ≥80% were brought into culture in a collagen-collagen sandwich. The primary mouse hepatocytes were allowed to recover for 40-42 hours at 37°C in a humidified chamber with 95%/5% air/CO_2 in serum-free culture medium supplemented with glucagon (7 ng/mL), insulin (0.5 U/mL), 2% penicillin/streptomycin (5000 U/mL penicillin and 5000 µg/mL streptomycin), and hydrocortisone (7.5 µg/mL). Culture medium was refreshed every 24 hours. After the recovery period, cells were exposed to culture medium containing CsA or 0.5% DMSO as a vehicle control. Based on IC_{20} values, which were determined by the MTT reduction method [10], hepatocytes were exposed as follows: 0.5% DMSO as a vehicle control and 10 µM CsA for IC_{20} at 48 hours. For transcriptomics analyses, three independent biological experiments, each with cells from a different animal, were performed. For metabolome analysis, five experiments were performed.

Transcriptomics

mRNA expression profiling

Standard procedures were used to perform total RNA isolation, target preparation and microarray hybridization on the Affymetrix Mouse Genome 430 2.0 arrays and scanning on an

Affymetrix GeneArray scanner. The Arrayanalysis.org web service was used for quality control [11] and all microarrays were of high quality. A total of 24 CEL files were re-annotated to EntrezGene IDs using the MBNI BrainArray Custom CDF-files v15.1 [12], RMA normalized [13] and the intensities were log2 transformed. Differentially expressed genes (DEGs) were selected using the combination of the following criteria: I) a p value <0.05 determined by a paired Student's T-test, II) for the three replicates an average absolute fold change (FC) of 1.5 or higher (i.e. average log2 ratio of < -0.58 or >0.58).

Pathway analysis using ConsensusPathDB [14] was used for biological interpretation of differentially expressed genes after treatment with 10 µM cyclosporin A for 48 hours. Over-representation analysis for pathways was performed using the mouse Entrez Gene IDs of the differentially expressed genes. Pathways were considered significantly affected if the q-value < 0.05 and if there was a minimal overlap of three genes with the input list. The q-values represent the p-values corrected for multiple testing using the False Discovery Rate (FDR) method.[15]

microRNA expression profiling

MicroRNA expression profiling was performed using Agilent Sureprint G3 Unrestricted Mouse miRNA V19 8 x 60K microarrays. The hybridization was performed following standard protocols, after which the microarray slides were washed and scanned with a DNA microarray scanner (Agilent Technologies). The scanned images were converted into TXT files using the Feature Extraction Software v10.7.3.1 from Agilent Technologies, which were imported in R 2.15.2 (http://www.r-project.org) for quality control with an in-house developed pipeline. Further downstream analysis was being performed by means of AgiMicroRna.[16] Total Gene Signals were log2-transformed and quantile normalized. The AgiMicroRna filtering procedure was based on subsets of experimental conditions (treated and matching control samples). The selection of differentially expressed microRNAs was identical to the indicated two criteria for mRNAs.

The transcriptomics data discussed in this publication have been deposited in NCBI's gene expression omnibus[17] and are accessible through GEO series accession number GSE55883.

Proteomics

Proteomics results from primary mouse hepatocytes exposed to CsA were derived from published experiments by Van Summeren et al.[18] In short, after exposure to CsA, amiodarone or acetaminophen, cells were washed and proteins were isolated. Labeling and differential in gel electrophoresis (DIGE) was performed and a one-way ANOVA test (p<0.05) was used to select the significant differential spots between the experimental groups. Differentially expressed proteins were identified using mass spectrometry. A post-hoc test was performed to find differentially changed proteins between CsA-treated cells and solvent controls.

Metabonomics

The polar and apolar metabolites were extracted from exposed cells and analysis with 1H-NMR was done as described previously.[19] The data were pre-processed and aligned using an in-house developed program.[20] The aligned fingerprint data were normalized using Probabilistic Quotient Normalization [21] combined with log2 transformation. Differentially expressed metabolites were selected using the combination of the following criteria: I) the Student's T-test p-value was <0.05, II) the absolute FC was >1.5 for all five biological replicates. Metabolites were identified by using a combination of commercial standards, literature and databases like the Human Metabolome Database. [22] Raw data of the polar and apolar peaks are accessible through Additional file 6 and 7, respectively.

Integrative data analysis

Selection of microRNA/mRNA targets

For the integration of microRNAs and their mRNA targets, first the database miRTarBase [23] was used to get a list of experimentally validated microRNA-target interactions *(Mus Musculus*; Release 4.4). Additionally, also predicted mRNA targets were of interest. For that purpose, custom Perl scripts were used to combine two flat-text files derived from miRanda [24] (release date: Aug 2010) and TargetScan [25] (release 6.0). The mouse 'good mirSVR score, Conserved miRNA' text file from miRanda and the 'Predicted Conserved Targets Info' file from TargetScan were merged based on the stable MIMAT identifier and can be downloaded at http://web.tgx.unimaas.nl/svn/public/miRNA/. As such, the merged file only contains highly conserved microRNA targets.

Network visualization of integrated data

For the biological interpretation of the significant proteins, a network was created using the MiMI-plugin [26] in Cytoscape (version 2.8.3). [27] The selected significant proteins were uploaded into the MiMI-plugin and a network was created based on known biological interactions including nearest neighbors. CyTargetLinker was used to add microRNAs to this network based on information from miRTarBase (version 4.4).

Results

Omics analyses

After exposure to CsA the gene, microRNA, protein and metabolite expressions of primary mouse hepatocytes were analyzed. Microarray-based transcriptomic analyses of primary mouse hepatocytes revealed 1836 differentially expressed genes for mRNA analysis of which 813 were up-regulated and 1023 were down-regulated (Additional file 1). Pathway analysis was performed for biological interpretation of differentially expressed genes, resulting in 223 significantly changed pathways which were allocated to general cellular processes (Table 1). 68 pathways, approximately 30% of the total number, were related to processes involved in lipid metabolism. Amino acid metabolism and drug metabolism were also significantly affected, representing approximately 14% and 9% of the significantly affected pathways, respectively. A complete overview of the significantly changed pathways, the general cellular processes and the differentially expressed genes involved can be found in Additional file 2.

7 differentially expressed microRNAs were found for microRNA analysis of which five were up-regulated and 2 down-regulated (Table 2). The five up-regulated microRNAs were mmu-miR-20a, mmu-miR-182, mmu-miR-183, mmu-miR-767, mmu-miR-3096b and the two down-regulated microRNAs were mmu-miR-212 and mmu-miR-221.

For the creation of molecular networks, the significantly changed proteins after CsA treatment were used, which were found with a post-hoc test between CsA-treated cells and the corresponding controls. This resulted in 21 differential protein-spots belonging to 19 unique proteins (Table 3).

Metabonomics analysis revealed 5 differentially expressed metabolites (Table 4). Analysis of polar metabolites showed significant up-regulation of glucose. Analysis of the apolar cell extracts revealed a decreased level of fatty acyl chains and an increase of free fatty acids, glycerol backbone and glycerophospholipid backbone.

Integrative data analysis

To investigate protein-mRNA-microRNA interactions after CsA exposure, proteomic and transcriptomic data were analyzed to find sets of differentially expressed proteins, genes and microRNAs. 19 differentially expressed proteins were found and 9 of these 19 genes were also found to be differentially expressed in the gene expression analysis after treatment with 10 µM CsA for 48 hours (Table 3). MicroRNA-mRNA-targets for these 9 genes were evaluated using experimentally validated data from miRTarBase, as well as the overlap of predicted data from TargetScan and MiRanda. However, no associated microRNAs were found using miRTarBase.

After integration, one set was found of which protein, gene and associated microRNA indeed appeared significantly expressed after the treatment with 10 µM CsA for 48 hours. The corresponding protein, member 4 from protein disulfide isomerase family A, was significantly up-regulated with a FC of 1.40. The PDIA4 gene coding for this protein was also significantly up-regulated with a FC of 1.84. Based on the merged list of MiRanda and TargetScan, mmu-miR-182 is predicted to target the PDIA4 mRNA. This microRNA was up-regulated with a FC of 2.04. Exploring the related Gene Ontology processes for this protein revealed a role in folding and secretion of proteins in the endoplasmic reticulum (ER).

Network visualization of integrated data

To further investigate the interaction between the significantly changed proteins and the associated genes and microRNAs, networks were created using Cytoscape. Firstly, the 19 significantly modulated proteins were up-loaded into the MiMI-plugin of Cytoscape and interactions were investigated including genes directly interacting with these proteins. Interactions were found for 8 of the 19 proteins including nearest neighboring genes (Figure 1). In total, 88 genes were added to the network; expression levels of 36 genes appeared significantly changed after treatment with CsA ($p<0.05$) and 23 of these significantly changed gene expressions had absolute FCs higher than 1.5. Six clusters could be distinguished, which were associated with transport (Cluster 1), cell cycle processes (Cluster 2), lipid homeostasis (Cluster 4), glycolysis (Cluster 5) and protein folding and catabolism (Cluster 3, 4 and 6). Secondly, experimentally validated microRNA/mRNA interactions were added using the CyTargeLinker-plugin of Cytoscape based on the miRTarBase database. 13 microRNAs were added to the network of which one, mmu-miR-221, appeared significantly expressed after CsA treatment with a FC of -1.55 (Figure 2). All significant proteins, genes and microRNAs were visualized in this network and all nodes included in this network are listed in Additional file 4.

Discussion

In this study we present an integrative cross-omics analysis of CsA-induced toxicity in primary mouse hepatocytes. Expression of proteins, genes, microRNAs and metabolites were analyzed after treatment with 10 µM CsA for 48 hours. The present study reveals that in total 1836 genes were significantly expressed after CsA treatment. Pathway analysis of mRNA results indicated changes in lipid, amino acid, drug, glucose and bile metabolism on transcription level, which can be related to drug-induced cholestasis. However, to elucidate the complete cellular response to CsA treatment, these transcriptomic changes need to be confirmed by measuring the protein and metabolite levels.

Protein results have previously been published by Van Summeren et al. and the authors concluded that the deregulated proteins were involved in ER stress and ER-Golgi transport. [18] In addition to mRNA results, proteomics investigation of CsA-induced toxicity reveals that processes like vesicle-mediated transport and protein secretion may be affected. MicroRNA expression profiling resulted in 7 differentially expressed microRNAs after CsA treatment. MiR-182 and miR-183 were differentially up-regulated and belong to the same microRNA-cluster. MiR-96 is also part of this microRNA-cluster and although this microRNA was also up-regulated, the change was not significant due to high inter-sample variation. The microRNAs in this cluster interact with genes involved in the insulin signaling pathway [28], which was shown to be linked to ER stress and the UPR. [29-30] Mir-212 was shown to target CYP2E1 and miR-212 expression was shown to be regulated by insulin [31], further emphasizing a link between insulin signaling and drug-induced ER stress. MiR-20a was shown to negatively regulate E2F1 expression, which is an important transcription factor involved in cell cycle progression. [32] MiR-221 was also shown to target mRNAs involved in pathways related to cell cycle progression and apoptosis. [33] No literature was found describing possible roles for miR-767. Furthermore, miR-3096b has been shown to be independent of Dicer and is believed to be a misannotation. [34] Additional to the insights gained from mRNA and protein analysis, microRNA analysis indicates that microRNAs involved in cell cycle progression and insulin signaling are differentially expressed, which both can be linked to drug-induced toxicity.

The differentially expressed proteins, genes and microRNAs were investigated to find sets of which the protein, gene and interacting microRNA(s) were all significantly affected. The protein disulfide isomerase family A, member 4 (Pdia4) coding gene and protein were differentially expressed after CsA treatment. Furthermore, the microRNA mmu-miR-182-5p, which is predicted to interact with the Pdia4 mRNA by both TargetScan and MiRanda, was also differentially expressed. Most studied are the mechanisms underlying microRNA-induced mRNA decay and repression of mRNA translation resulting in repressed protein synthesis. [35-37]

However, the up-regulated expression of Pdia4 mRNA is accompanied with an up-regulated protein expression, while the mmu-miR-182-5p is also up-regulated. A clear repression on the translation of the Pdia4 mRNA by mmu-miR-182-5p could therefore not be found in our experiments. Interestingly, an emerging number of studies show that microRNAs are also able to activate translation of target-mRNAs in quiescent cells. [38-39] Since primary mouse hepatocytes are not dividing in culture and thus are in a quiescent state, microRNA-induced translation could also affect mRNA and protein levels. Future research should point out if there is an actual interaction between miR-182 and the Pdia4 mRNA and, if so, how this interaction influences the expression of the Pdia4 mRNA and protein. However, the significant changes found after CsA treatment for the expression of the Pdia4 mRNA and protein and the predicted interacting microRNA suggest a possible role in the CsA-induced mechanisms underlying drug-induced hepatotoxicity.

Apart from the directionality of effect from microRNAs on the levels of mRNAs and proteins, different time- and dose-dependent effects on the expression of mRNAs, microRNAs and proteins may play a role. Therefore, investigation of the expression of the mRNAs and microRNAs was performed at an earlier time point, 24 hours, and using a higher concentration, 50 μM, which is the IC_{20} at 24 hours based on the MTT reduction assay (Additional file 5). This analysis demonstrated that the arfaptin-1 protein expression was significantly lower after CsA treatment for 48 hours, while the gene was not differentially expressed at this time point. Investigating the expression of this gene after 24 hours of treatment showed a significant up-regulation. Three microRNAs interacting with the mRNA of this gene were found, however, no differential expression of these microRNAs was detected after treatment with 10 μM CsA for 24 and 48 hours. Increasing the dose to 50 μM CsA and incubating the cells for 48 hours resulted in more significantly expressed microRNAs, including the three microRNAs interacting with the arfaptin-1 mRNA. Eight genes coding for proteins significantly affected after CsA treatment were differentially expressed after treatment with 10 μM for 48 hours, however, no interacting microRNAs were found or the interacting microRNAs were not differentially expressed with this dose. The high dose treatment resulted in differential expression of microRNAs interacting with three of these genes. In total 4 more sets were found where mRNA, protein and interacting microRNA(s) expression were significantly affected in at least one of the treatments, namely arfaptin-1, heat shock 70 kDa protein 5, protein disulfide-isomerase, and protein disulfide-isomerase family A, member 3 (Additional file 3). These proteins are all involved in protein folding and secretion, further emphasizing the unfolded protein response as an important mechanism in CsA-induced hepatotoxicity. Since hepatotoxicity is a dynamic process, dose dependency studies, but also time series analyses are important to fully elucidate the mechanisms underlying drug-induced hepatotoxicity. A combination of such studies in PMH with high-throughput omics analyses would be a powerful tool to study the mechanisms underlying drug-induced liver injury.

To further investigate CsA-induced mechanisms, a network was created using the significantly changed proteins and their neighboring genes. This resulted in five clusters which are involved in several cellular processes like cell cycle, amino acid homeostasis, glucose homeostasis, lipid homeostasis and transport. Furthermore, three of these clusters were involved in important processes involved in the UPR, namely protein folding and protein catabolism. The network was further expanded with interacting microRNAs. Overall, all proteins, genes and microRNAs in the expanded network are involved in processes known to be affected after CsA treatment, suggesting a possible role in drug-induced toxicity.

Metabolites were analyzed to further elucidate the mechanisms underlying CsA-induced hepatotoxicity. Investigation of the polar metabolites after treatment with 10 μM resulted in 1 significantly up-regulated metabolite, glucose. This higher expression of glucose after CsA treatment could possibly be explained by down-regulation of glycolysis, while one of the significantly down-regulated proteins was α-enolase. No significantly expressed microRNAs were predicted to interact with the mRNA of α-enolase and no mRNA levels were assessed with the used arrays, so regulation of this protein could not be further investigated in our experiments. However, in cluster 5 of the expanded network more genes involved in glucose homeostasis and microRNAs interacting with the mRNA of these genes are visible and could be involved in the down-regulation of glycolysis. One of these genes is pyruvate kinase and the mRNA coding for this enzyme was significantly down-regulated after CsA treatment. It has been shown that fatty acids are capable of down-regulating pyruvate kinase; investigation of the apolar metabolites indeed shows a significant increase of the fatty acids level after CsA treatment. The level of glycerol is also increased after CsA treatment. However, the level of fatty acyl chain is decreased. A possible explanation is that the long chain fatty acids are still broken down by beta-oxidation in the peroxisomes, resulting in a decreased level of fatty acyl chain, but the short chain fatty acids are not further oxidized in the mitochondria due to mitochondrial stress, thus resulting in increased levels of free fatty acids and glycerol.

When investigating apolar metabolites of the samples treated with 50 μM CsA, total cholesterol levels were decreased after 48 hours, where the level of esterified cholesterol was increased. Furthermore, after treatment with the high dose, levels of sphingomyelin, choline and multiple cholesterol protons were decreased. The detection of several other apolar metabolites was impossible because of interference from CsA. Protons belonging to CsA were detected after treatment with the high dose, suggesting an intracellular accumulation of CsA. Sphingomyelin is synthesized by the ER and the sphingomyelin level was lower after CsA treatment which could therefore be a result of the CsA-induced ER stress. Kockx et al. showed that cholesterol accumulation can inhibit ER to Golgi transport and protein secretion. [40] Total cholesterol levels and peaks belonging to multiple cholesterol protons are decreased after CsA treatment; however, the level of esterified cholesterol is increased after treatment with the high dose for 48 hours. Unfortunately, levels of free cholesterol could not be investigated because of interference by the protons belonging to CsA. A relative increase of the esterified fraction of cholesterol could be an indication that the cells are trying to secrete the intracellular cholesterol via lipoproteins as a reaction to reduced bile acid flow. This would explain the total amount of cholesterol decreasing, while the esterified fraction increases. Overall, additional to mRNA, microRNA and protein analyses, metabonomic investigation of CsA-induced toxicity reveals impairment of the lipid and cholesterol homeostasis. These processes can be related

to cholestatic mechanisms, which would indicate CsA-induced cholestasis in primary mouse hepatocytes.

Although single omics analysis can indicate which cellular processes are affected after exposure to toxic compounds, integrating multiple omics reveals the implications of these compound-induced changes and further elucidates the mechanisms underlying compound-induced toxicity.

The AOP for cholestasis recently published by Vinken et al. summarizes the literature on the deteriorative and adaptive cellular response induced by bile acid accumulation as a result of BSEP inhibition. Many of the cellular responses described in this AOP were also found to be affected in our study on PMH after exposure to CsA, which is a known inhibitor of the BSEP. However, integrating multiple omics additionally identified the involvement of ER stress and the UPR in CsA-induced hepatotoxicity. Activation of several nuclear receptors is indicated to be a key event in the AOP for cholestasis, which in turn results in altered gene expression of target genes. Since the ER is important in the translation and folding of proteins, induced transcriptomic changes by nuclear receptors are dependent on a correct functioning of the ER. This novel finding resulting from an innovative multi-omics integration effort, thus indicates that ER stress represents an important process in the induction of cholestasis and should be included in an AOP for cholestasis.

Conclusions

This integrative omics analysis of the transcriptome and the proteome in primary mouse hepatocytes further emphasizes the induction of ER-stress and the unfolded protein response by cyclosporin A. microRNA analysis indicates that cell cycle progression and insulin signaling are involved in CsA-induced toxicity. Furthermore, the metabonomics and transcriptomics analysis revealed impairment of the lipid and cholesterol homeostasis which may be a result of mechanisms involved in drug-induced cholestasis. We conclude that cross-omics analysis confirms and strengthens the results found with the single omics approach and has the potential of substantially deepening our insights in Adverse Outcome Pathways.

Availability of supporting data

The data sets supporting the results of this article are included within the article (and its additional files) or are available in the NCBI's gene expression omnibus. The transcriptomics data are accessible through GEO series accession number GSE55883.

Acknowledgements

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Table 1. *Cellular processes affected after treatment with 10 µM cyclosporin A for 48 hours based on differentially expressed genes. ConsensusPathDB pathways that were significantly affected were allocated to general cellular processes.*

Cellular Process	# of Pathways
Lipid Metabolism	68
Amino Acid Metabolism	32
Drug Metabolism	20
Glucose Metabolism	13
Complement System	11
Hemostasis	10
Protein processing	9
Bile Metabolism	8
Cell Surface Interactions	2
Apoptosis	2
Peroxisome	2
Other Cellular Processes	46
Total	223

Table 2. *Differentially expressed microRNAs (absolute FC > 1.5 and p-value < 0.05) of primary mouse hepatocytes after treatment with 10 µM cyclosporin A for 48 hours with their FC and p-value.*

microRNA	FC	p-value
mmu-miR-182-5p	2.04	0.04
mmu-miR-183-5p	2.07	0.00
mmu-miR-20a-5p	1.63	0.05
mmu-miR-212-3p	-1.55	0.01
mmu-miR-221-3p	-1.55	0.03
mmu-miR-3096b-3p	1.53	0.02
mmu-miR-767	3.5	0.01

Table 3. *Protein and gene expression results after treatment with 10 µM cyclosporin A for 48 hours for the significantly changed proteins.*

Protein name	FC protein	p-value protein	Gene name	FC gene	p-value gene
Protein disulfide-isomerase	*1.89*	*0.01*	*P4hb*	*1.64*	*0.00*
78 kDa glucose-regulated protein	*1.69*	*0.01*	*Hspa5*	*2.08*	*0.00*
Hydroxyacylglutathione hydrolase, mitochondrial	*1.24*	*0.03*	*Hagh*	*1.40*	*0.00*
Mesencephalic astrocyte-derived neurotrophic factor	*1.63*	*0.00*	*Manf*	*3.56*	*0.01*
Peptidyl-prolyl cis-trans isomerase B / Cyclophilin B	*-1.68*	*0.00*	*Ppib*	*1.19*	*0.02*
Peptidyl-prolyl cis-trans isomerase B / Cyclophilin B	*-1.90*	*0.00*	*Ppib*	*1.19*	*0.02*
Protein disulfide isomerase A4	*1.40*	*0.01*	*Pdia4*	*1.84*	*0.02*
Keratin, type I cytoskeletal 10	*1.35*	*0.01*	*Krt10*	*1.61*	*0.03*
Ornithine carbamoyltransferase, mitochondrial	*2.12*	*0.01*	*Otc*	*-6.60*	*0.03*
Liver carboxylesterase 1	*-1.46*	*0.04*	*Ces1*	*-6.26*	*0.04*
Proteasome subunit beta type-7	*1.22*	*0.02*	*Psmb7*	*1.12*	*0.06*
Serum albumin precursor	*1.86*	*0.01*	*Alb*	*-1.07*	*0.07*
Protein disulfide-isomerase A3	*-1.46*	*0.04*	*Pdia3*	*1.46*	*0.07*
Isoform 1 of Peroxisomal coenzyme A diphosphatase NUDT7	*1.22*	*0.02*	*Nudt7*	*-1.38*	*0.12*
Indolethylamine N-methyltransferase	*1.54*	*0.04*	*Inmt*	*-1.26*	*0.18*
Perilipin-2 / Adipophilin	*-2.01*	*0.00*	*Plin2*	*-1.15*	*0.18*
Perilipin-2 / Adipophilin	*-2.25*	*0.00*	*Plin2*	*-1.15*	*0.18*
Voltage-dependent anion-selective channel protein 2	*1.24*	*0.01*	*Vdac2*	*1.04*	*0.51*
Proline-rich protein 15	*1.63*	*0.00*	*Prr15*	*-1.03*	*0.70*
Arfaptin-1 protein	*-2.25*	*0.00*	*Arfip1*	*-1.01*	*0.81*
LOC100044223 Alpha-enolase	*-2.25*	*0.00*	*Eno1*	*NA*	*NA*

Table 4. *Differentially expressed metabolites in primary mouse hepatocytes after exposure to 10 µM CsA for 48 hours with their FCs.*

Polar Metabolites	FC
Glucose	*2.43*
Apolar Metabolites	
Fatty acyl chain	*-1.62*
Free fatty acids	*4.87*
Glycerol Backbone	*3.59*
Glycerol/Glycerophospholipid Backbone	*3.79*

NA, Not Analyzed; Adapted from Van Summeren, A., et al.[18]

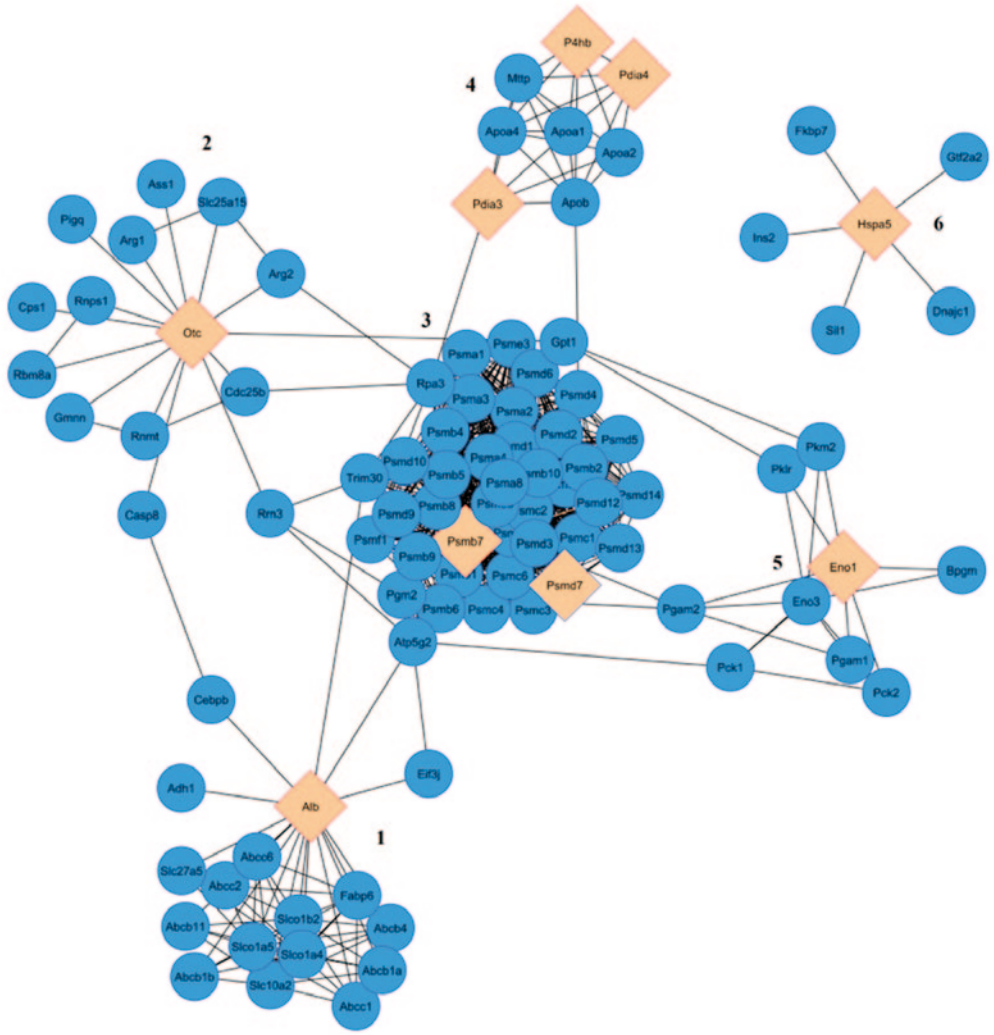

Figure 1. *Network created with Cytoscape to visualize the interaction between the significantly changed proteins (diamonds) and their interactions with neighboring genes (circles) resulting in 6 clusters.*

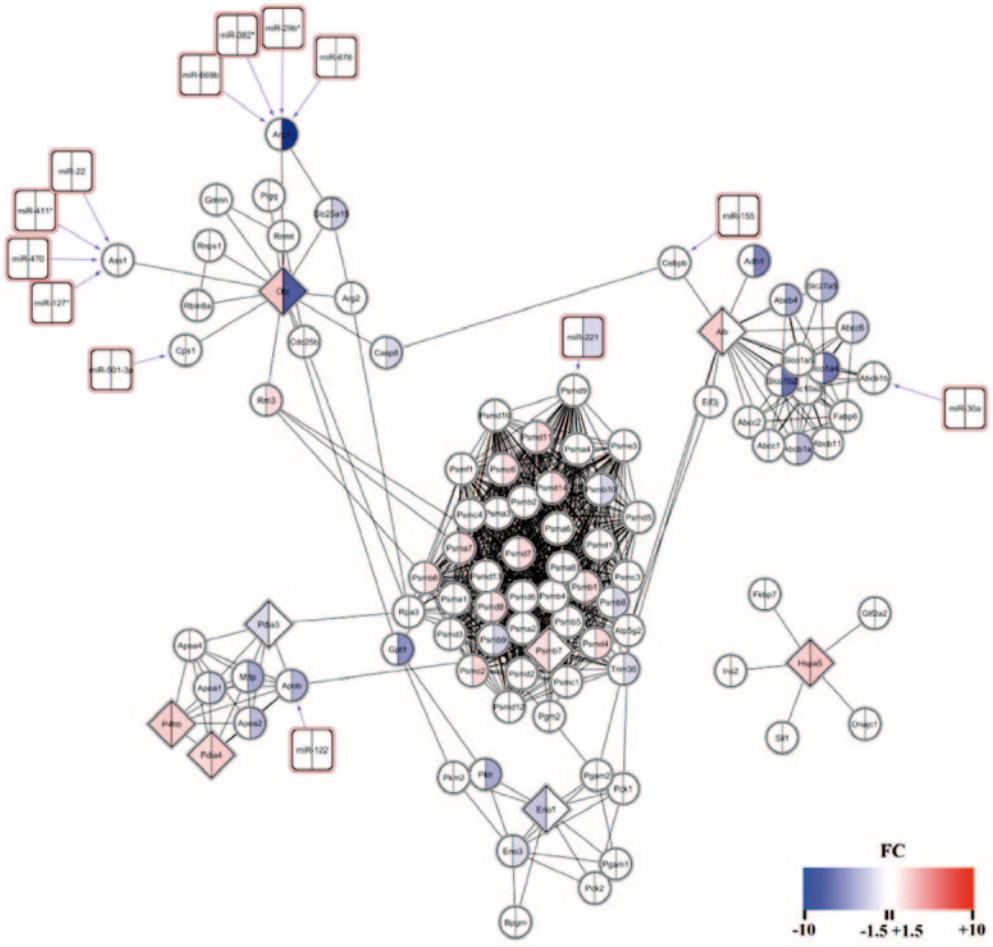

Figure 2. *Network created with Cytoscape to visualize the interaction between the significantly changed proteins (diamonds) and their interactions with neighboring genes (circles) and validated interacting microRNAs based on miRTarBase database (squares). Significant proteomics data is visualized on the left side of the nodes and significant gene and microRNA data on the right side of the nodes, based on the fold changes after treatment with 10 µM cyclosporin A for 48 hours.*

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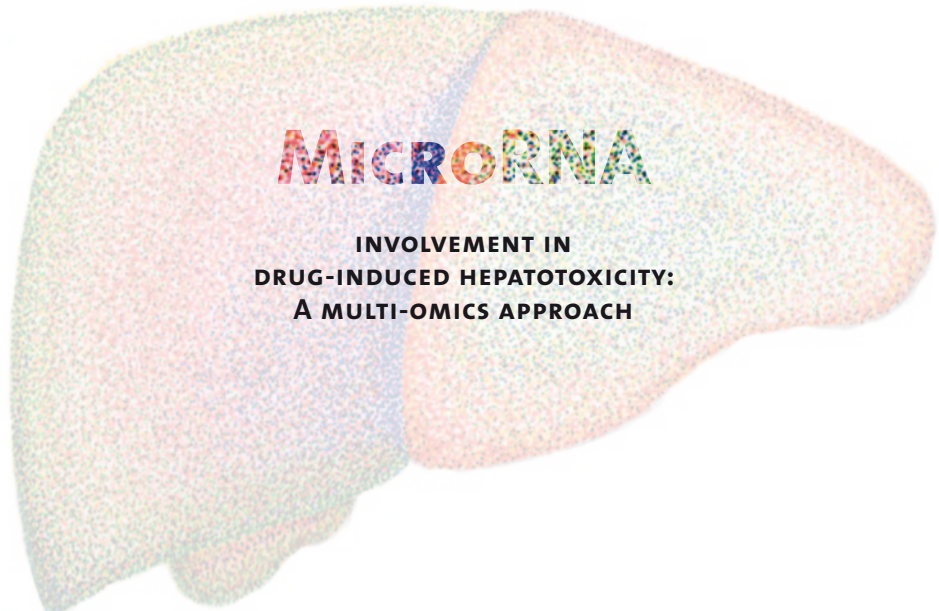
Chapter 6



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Abstract

Drug-induced liver injury is a frequently reported reason for project termination during drug development and withdrawal of drugs already available on the market. Therefore, early detection of drug-induced toxicity is important. Liver specific microRNAs have been proposed as early biomarkers of hepatotoxicity; however their role in drug-induced toxicity is largely unknown. We investigated the microRNA and mRNA expression changes induced *in vitro* by three well known hepatotoxicants. HepG2 cells and primary mouse hepatocytes were treated with acetaminophen, amiodarone and cyclosporin A and RNA was isolated. Microarrays were performed to investigate the hepatotoxicant-induced changes. Different significantly expressed microRNAs are observed in HepG2 cells and primary mouse hepatocytes. MiR-2110 and miR-3152 are differentially expressed in HepG2 cells after treatment with all three compounds for 24 hours. In primary mouse hepatocytes miR-212 and miR-3470a are overlapping after treatment for 24 hours with these three compounds. Investigation of these microRNAs and their targets indicates a possible role in regeneration and proliferation, which may induce cell repair and inhibit apoptosis. Furthermore, drug-specific changes in microRNA expression levels are observed in both cell models. Investigation of these drug-specific differentially expressed microRNAs suggests a possible role in the processes involved in liver injury. MicroRNAs targeting genes involved in lipid metabolism are differentially expressed in multiple treatments. Furthermore, changed microRNAs in *in vivo* cholestasis are differentially expressed after CsA treatment in primary mouse hepatocytes. Targeted research of these drug-specific microRNAs including their target genes and the proteins they code for may further unravel the mechanisms underlying drug-induced hepatotoxicity.

Introduction

Drugs are mostly metabolized and secreted via the liver, making this organ vulnerable for adverse drug reactions. Drug-induced hepatotoxicity is one of the most indicated reasons for drug withdrawal during clinical trials or after admittance onto the market. Early detection of liver damage is crucial and biomarkers of hepatotoxicity have been investigated extensively. In the search for new early biomarkers in serum of patients suffering from liver damage, several microRNAs have been suggested to present specific for liver which could be used to detect liver injury. The most described microRNA is miR-122, which is the predominant microRNA in the liver. [1-2] Increased circulating levels of miR-122 have been suggested to be a specific biomarker for several forms of liver injury, including necrosis, steatosis and cholestasis, even outperforming the classical biomarker alanine transaminase. [3-6] Although microRNAs have been suggested as potential biomarkers for liver injury, their role in the mechanisms underlying drug-induced hepatotoxicity is largely unknown.

MicroRNAs are mostly known for their post-transcriptional regulation of gene expression by interacting with the mRNA and promoting its degradation or inhibiting its translation. However, other literature indicate a possible promotion of target mRNA translation in quiescent cells. [7-8] Overall, their exact role in biology is not fully understood. MicroRNAs are believed to interact with multiple targets, even up to several hundred genes. [9] Several databases are based on prediction algorithms that calculate possible microRNA targets based on their sequence. The miRTarBase database is based on experimentally validated targets described in literature. [10] Interactions of microRNAs and mRNAs are being investigated in many fields of research, including drug-induced toxicity. However, the number of publications regarding the role of microRNAs in drug-induced hepatotoxicity is limited.

We therefore aimed to investigate changes on expression of microRNAs by drugs known to induce hepatotoxicity and the regulation of their experimentally validated target genes. Two *in vitro* liver models, HepG2 cells and primary mouse hepatocytes, were treated with three well known hepatotoxicants, namely cyclosporin A, amiodarone and acetaminophen. HepG2 cells have frequently been applied in drug toxicity research and their easy availability and culture conditions make them a straightforward model for human *in vitro* toxicity screening. However, HepG2 cells have lost several important liver specific functions. Primary mouse hepatocytes cultured in a collagen sandwich configuration maintain their metabolic competence and since most drug toxicity screenings are performed in rodents, it is proposed as a relevant *in vitro* liver model. [11]

Materials and methods

Chemicals

Modified Eagle's medium (MEM) plus glutamax, fetal calf serum (FCS), non-essential amino acids, sodium pyruvate, penicillin/streptomycin, insulin and Hanks' calcium- and magnesium-free buffer were purchased from Invitrogen (Breda, The Netherlands). Glucagon, hydrocortisone (water soluble), collagenase type IV, dimethylsulfoxide (DMSO), N,N-dimethylformamide (anhydrous, 99.8%), NaCl, $NaHCO_3$, KCl, KH_2PO_4, $MgSO_4$, glucose, $CaCl_2$, Acetaminophen, Amiodarone, and Cyclosporin A were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Collagen type I Rat Tail was obtained from BD Biosciences (Bedford, MA, USA). The Trizol reagent and the RNeasy mini kit were from Qiagen Westburg (Leusden, The Netherlands).

HepG2 data

HepG2 cells were cultured in MEM plus glutamax containing 10% v/v FCS, 1% v/v Sodium Pyruvate, 1% v/v non-essential amino acids, 2% w/v penicillin and streptomycin at 37°C in an atmosphere containing 5% CO_2.

Cells were seeded in 6 well plates and exposed to Cyclosporin A, Amiodarone, Acetaminophen and matching controls (0.5% v/v DMSO) for 24 hours. The IC_{20} of 24 hours was used to treat the cells. Concentrations were as follows: Cyclosporin A (CsA), 20 µM; Amiodarone (AM), 15 µM; Acetaminophen (APAP), 10 mM.

mRNA microarray analysis

Total RNA isolation, target preparation and microarray hybridization on the Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays and scanning on an Affymetrix GeneArray scanner, were performed according to standard procedures. Quality control was performed using the Arrayanalysis.org web service and all microarrays were of high quality. [12] CEL files were re-annotated to EntrezGene IDs using the MBNI BrainArray Custom CDF-files v17.1.0 [13], RMA normalized combined with MAS5-PMA-calling [14] and the intensities were log2 transformed.

microRNA microarray analysis

MicroRNA expression profiling was performed using sixth generation miRCURY LNA microRNA arrays (Exiqon, Vedbaek, Denmark). Hybridization and scanning with a DNA microarray scanner (Agilent Technologies) were performed following standard protocols. Feature Extraction Software v10.7.3.1 from Agilent Technologies was used to convert the scanned images into TXT files, which were imported in R 2.15.2 (http://www.r-project.org) for quality control and intensities were log2-transformed and quantile normalized.

Primary mouse hepatocytes data

The animal studies were approved by the Animal Ethical Committee of the Maastricht University, The Netherlands (approval number: 2011-108). Adult male C57BL/6 mice, weighing 20-25 grams, were obtained from Charles River GmbH, Sulzfeld, Germany. Animals were housed in macrolon cages with sawdust bedding at 22°C and 50–60% humidity. Food and tap water were available *ad libitum*, and the light cycle was 12 hours light/12 hours dark. Isolation and culture of primary mouse hepatocytes were performed as described before. [11] After a recovery period, cells were exposed to culture medium containing Cyclosporin A, Amiodarone, Acetaminophen or 0.5% v/v DMSO as a vehicle control. Based on the IC_{20} value for 24 hours, the hepatocytes were exposed as follows: 0.5% v/v DMSO as a vehicle control; CsA: 50 µM; AM: 25 µM; APAP: 10 mM.

mRNA microarray analysis

Standard procedures were used to perform total RNA isolation, target preparation and microarray hybridization on the Affymetrix Mouse Genome 430 2.0 arrays and scanning on an Affymetrix GeneArray scanner. The Arrayanalysis.org web service was used for quality control and all microarrays were of high quality. CEL files were re-annotated to EntrezGene IDs using the MBNI BrainArray Custom CDF-files v17.1.0, RMA normalized and the intensities were log2 transformed.

microRNA microarray analysis

MicroRNA expression profiling was performed using Agilent Sureprint G3 Unrestricted Mouse miRNA V19 8 x 60K microarrays. The hybridization was performed following standard protocols, after which the microarray slides were washed and scanned with a DNA microarray scanner (Agilent Technologies). The scanned images were converted into TXT files using the Feature Extraction Software v10.7.3.1 from Agilent Technologies, and were imported in R 2.15.2 (http://www.r-project.org) for quality control with an in-house developed pipeline. Further downstream analysis was being performed by means of AgiMicroRna. [15] Total Gene Signals were log2-transformed and quantile normalized.

Statistical analyses

Features that passed the Quality Controls were used as input for analysis of differentially expressed genes and microRNAs using the BioConductor package LIMMA version 3.18.3. [16] First, for each of the experiments a linear model was fitted to the expression data, whereby replicate information (pairing) was treated as random effect. Subsequently, contrasts were defined that estimated the compound effect over DMSO controls. A moderated t-test was executed to find differentially expressed genes and microRNAs based on a combination of a False Discovery Rate corrected P-value < 0.05, and an average absolute fold change (FC) of 1.5 or higher. Venn diagrams created with VENNY [17] were used to investigate the overlap of differentially expressed genes and microRNAs between the different treatments.

Selection of microRNA/mRNA targets

For the integration of microRNAs and their mRNA targets, the database miRTarBase [10] was used to get lists of experimentally validated microRNA-target interactions (Release 4.5).

Pathway analysis

Lists of differentially expressed genes and target genes of differentially expressed microRNAs were uploaded into the web-tool ConsensusPathDB for human and mouse pathway over-representation analysis. [18] Pathways were considered significantly affected if the False Discovery Rate (FDR) was <0.05 and the percentage of input genes of the total number of genes in a pathway was ≥10%. Pathways with less than three genes were filtered out.

Results

Transcriptomic changes induced by cyclosporin A, amiodarone and acetaminophen

mRNA and microRNA expression profiling was performed of HepG2 cells and PMH exposed to CsA, AM and APAP. A moderated T-test was performed to minimalize the chance of finding false discoveries; however this stringent approach resulted in low number of significant genes and microRNAs in several exposures (Table 1). HepG2 cells exposed to CsA, AM and APAP, revealed 3217 differentially expressed genes (DEGs) for CsA and 2273 DEGS for APAP, where only 5 DEGs were found after AM treatment. CsA also induced the most significant changes at the microRNA level. Where APAP induced many significant changes at the mRNA level, only 4 microRNAs were significantly changed. AM treatment only induced 5 DEGs, but did result in 19 differentially expressed microRNAs. PMH treated with CsA, AM and APAP resulted in over 1000 DEGs for all three treatments, with up to 3972 DEGs for CsA treatment. Interestingly, AM induced the lowest number of DEGs but the second highest number of differentially expressed microRNAs.

Pathway analysis of the differentially expressed genes revealed overlapping processes between all three compounds (Additional files). AM treatment of HepG2 cells only induced 5 significantly changed genes and no differential pathways. CsA and APAP exposure in HepG2 cells both showed significant pathways involved in lipid, bile and glucose metabolism. CsA treatment also induced significant changes of the glutathione metabolism. Furthermore, most significant pathways were involved in cell cycle processes. In PMH, all three compounds induced changes in the lipid, bile and glutathione metabolism and significant pathways involved in glucose metabolism were found after CsA treatment. Moreover, one pathway involved in p53 signaling was significant after CsA and APAP treatment and no pathways involved in cell cycle processes were found for the three treatments in PMH.

Figure 1 A and B show respectively the overlap of up-regulated and down-regulated DEGs in HepG2 cells after treatment with CsA, AM, and APAP for 24 hours. In HepG2 cells only 1 gene, coding for sialidase 1, was significantly up-regulated in all three treatments, which was mainly due to the low response on gene expression level after AM treatment. In PMH the overlap between the different treatments was much higher (Figure 1 C and D). Moreover, treatment-specific genes were found for all treatments. Figure 2 shows the overlap of up-regulated and down-regulated differentially expressed microRNAs in HepG2 cells and PMH. Although some overlap was found in both HepG2 cells and PMH, most differentially expressed microRNAs were only significantly affected in one of the treatments. Table 2 shows the significantly down-regulated and up-regulated microRNAs in HepG2 cells (Table 2.1) and PMH (Table 2.2) after treatment with CsA, AM and APAP for 24 hours. Overall, the number of DEGs and differentially expressed microRNAs after treatment with CsA, AM and APAP did not appear to correspond and large differences between compounds were observed.

Interactions between significant microRNAs and their target genes after treatment for 24 hours in HepG2 cells

Several drug-specific microRNAs were differentially expressed and two microRNAs were significantly down-regulated in all three treatments, namely miR-2110 and miR-3152.

Experimentally validated interactions were investigated using lists from miRTarBase en revealed 4 target genes for miR-2110 and no interactions for miR-3152. The four target genes of miR-2110 are genes coding for chromosome 10 open reading frame 118 (C10orf118), bromodomain containing 4 (BRD4), nuclear receptor corepressor 2 (NCOR2) and fatty acid synthase (FASN). The genes C10orf118 and BRD4 were significantly up-regulated after treatment with CsA, but not with the other compounds. NCOR2 was not differentially expressed in any of the treatments and the expression of FASN was not assessed with the used microarrays.

CsA treatment in HepG2 cells for 24 hours resulted in 19 differentially expressed microRNAs which were investigated for target genes, based on the experimentally validate interaction list from miRTarBase. For 12 microRNAs no interactions were identified and for the remaining 7 microRNAs, in total 80 unique target genes were found. 11 target genes were not assessed with the used microarrays, 44 were not significantly changed and 25 were differentially expressed after CsA treatment. Of the 25 differentially expressed target genes, 10 were significantly up-regulated and thus showing a correlating response to the down-regulated microRNAs. The 10 correlating target genes are VCL, TIMP2, NOB1, HIF1A, NOP2, CDKN1A, BRD4, C10orf118, SHB, and IKZF5. The FXN gene showed interactions with two of the significantly down-regulated microRNAs, namely miR-559 and miR-935. However, the FXN mRNA was not differentially expressed after CsA treatment. Pathway analysis of all target genes revealed 2 significant pathways, Angiogenesis and HIF-2-alpha transcription factor network (Table 3).

16 microRNAs were differentially expressed in HepG2 cells treated with AM for 24 hours of which 7 showed interactions with 224 target genes. 31 of the target genes were not assessed and the remaining 193 were not significantly changed after treatment with AM. The PRRC2B and FASN genes were both targeted by two microRNAs. The PRRC2B gene was not significantly changed after AM treatment and was targeted by miR-324 and miR-1284, which were both significantly up-regulated. The expression of the FASN gene was not assessed and was targeted by miR-324 and miR-2110. MiR-2110 was significantly down-regulated after AM exposure. Pathway analysis of all target genes resulted in 28 significant pathways (Table 3).

APAP treatment in HepG2 cells for 24 hours revealed 4 differentially expressed microRNAs, 2 of which showed interactions with in total 200 target genes. 22 of these target genes were not assessed, 149 were not differentially expressed and 29 were significantly changed after APAP treatment. 12 target genes of the significantly up-regulated microRNA miR-183 were significantly down-regulated after APAP treatment. These correlating DEGs are TUBB1, SLC2A3, AUH, CCNB1, PPRC1, SRSF2, HNRNPM, SRSF10, MYBBP1A, C10orf2, TSR1, and GNL3. Pathway analysis of all target genes did not result in any significant pathways.

Interactions between significant microRNAs and their target genes after treatment for 24 hours in PMH

The overlap of differentially expressed microRNAs in PMH after treatment with CsA, AM, and APAP for 24 hours was visualized in Figure 2. Two microRNAs were differentially expressed in the same direction in all three treatments. MiR-212 was significantly down-regulated in all three treatments and one experimentally validated target gene was identified, namely Mmp9, for which expression was not assessed with the used microarrays. MiR-3470a was significantly up-regulated in all three treatments and no experimentally validated target genes were found for this microRNA.

CsA treatment for 24 hours in PMH resulted in 41 differentially expressed microRNAs of which 15 showed interactions with 721 target genes in total. 196 of these target genes were not assessed with the used microarrays, 335 were not differentially expressed and 190 were significantly changed after CsA treatment. 103 of the 190 differentially expressed target genes were significantly up-regulated and showed a correlating response to the interacting microRNAs, which were down-regulated. The gene Peli1 was not differentially expressed after CsA treatment and was shown to interact with 3 microRNAs: miR-21a, miR-30e and miR-425, which were all significantly down-regulated after CsA treatment. 47 genes showed interactions with two microRNAs of which 43 were shown to interact with both miR-30e and miR-425. 12 of these target genes were differentially expressed after CsA treatment, 19 were not significantly changed and 12 were not assessed. The Ddit4 gene was significantly down-regulated and shown to interact with miR-30e and miR-221, which were both down-regulated. The gene Taok1 was not significantly changed and interacts with the down-regulated miR-425 and miR-183. The Zfp281 gene was not differentially expressed and interacts with miR-425 and miR-203, which were both down-regulated. The Camk2a gene expression was not assessed and shown to interact with the down-regulated miR-148b and miR-152. Pathway analysis of all target genes resulted in 115 significant pathways (Table 4).

40 microRNAs were differentially expressed in PMH after treatment with AM for 24 hours. In total 439 target genes were identified for 11 microRNAs, of which 126 were not assessed, 280 were not significantly changed and 33 were differentially expressed after treatment with AM. 19 of the 33 differentially expressed target genes were down-regulated and correlating to the down-regulated interacting microRNAs. These correlating target genes were Ctgf, Megf9, Arrdc3, Slc30a10, Sbk1, Fgf13, Pik3r1, Cxcl12, Atp1b1, Tbcel, Slc35e2, Tmem2, Nsmf, Mlec, Gucd1, Ank3, Dpysl3, Abhd14b, and Pdcd4. 5 genes were targeted by two microRNAs: Foxp1 by miR-128 and miR-425, Tor1aip2 by miR-140 and miR-425, Taok1 by miR-183 and miR-425 Pten by miR-20a and miR-21a Peli1 by miR-21a and miR-425. These 6 microRNAs were all significantly up-regulated after AM treatment and the target genes were not differentially expressed. Pathway analysis of all target genes revealed 39 significant pathways (Table 4).

APAP exposure of PMH resulted in 23 differentially expressed microRNAs after 24 hours. 2 microRNAs were shown to have one target gene each. MiR-212 was significantly down-regulated and shown to target Mmp9, which was not assessed with the used microarrays. MiR-185 was significantly up-regulated after APAP treatment and shown to target Kdm6b, which was not differentially expressed. Pathway analysis of these 2 target genes did not result in any significant pathways.

Discussion

Drug-induced gene expression changes have been extensively investigated to unravel the mechanisms underlying drug-induced liver injury. However, drug-induced changes of microRNA expression and their influence on gene expression in drug-induced toxicity are largely unknown. In this study we investigate the significant changes in microRNA expression in HepG2 cells and PMH after treatment with the hepatotoxic compounds CsA, AM, and APAP. Several microRNAs were found to be significantly affected in all three treatments, suggesting a possible role in drug-induced liver injury.

MiR-2110 and miR-3152 were both significantly down-regulated in HepG2 cells after exposure to CsA, AM, and APAP for 24 hours. No target genes are experimentally validated for miR-3152 and no literature is available describing the function of this microRNA. Four genes are known to be targeted by miR-2110, namely BRD4, C10orf118, NCOR2 and FASN. Expression levels of BRD4 and C10orf118 are not affected after treatment with AM and APAP; however, expression of BRD4 and C10orf118 is significantly up-regulated after treatment with CsA, which is in line with the decreased expression of miR-2110. A study in mice showed that the protein Brd4 is involved in the regulation of transcription and proliferation. [19] Promotion of proliferation could be an adaptive response of hepatocytes to liver injury in order to repair the damaged tissue. Corresponding results were found in drug-induced hepatotoxicity in rats, where miR-122 was down-regulated after treatment with hepatotoxic compounds, which resulted in the up-regulation of target genes involved in cell proliferation. [20] The NCOR2 protein is a nuclear receptor co-repressor and is involved in down-regulating the expression of certain target genes. The NCOR2 gene was not differentially expressed in any of the treatments. The FASN gene encodes the Fatty Acid Synthase protein which catalyzes fatty acid synthesis. Overexpression of this gene can result in increased fatty acid synthesis and intracellular fatty acid accumulation. Accumulation of fatty acids can be an important phenotypical change in hepatotoxicity, particularly in steatosis. Since pathways involved in lipid metabolism were found in all three treatments, this microRNA could be involved in the alterations of lipid homeostasis in drug-induced toxicity. However, the expression of the FASN gene was not measured with the used microarrays.

Two microRNAs are differentially expressed in PMH after exposure to CsA, AM, and APAP. MiR-212 is significantly down-regulated in all three treatments and is shown to be involved in cell proliferation. Overexpression of miR-212 was shown to promote cell cycle progression and cell proliferation in non-small cell lung cancer cells [21]; however, a decreased cell proliferation was observed in gastric cancer cells when miR-212 was overexpressed [22], indicating the complexity of microRNA regulation. Based on the experimentally validated target list from miRTarBase, miR-212 shows interaction with the Mmp9 gene. Overexpression of the Mmp9 gene is observed during liver regeneration in mice [23], further emphasizing the possible role of miR-212 in liver cell proliferation. However, the expression of Mmp9 is not measured with the used mRNA microarrays. MiR-3470a is significantly up-regulated in PMH after treatment with CsA, AM, and APAP. No experimentally validated target genes are identified up till now and literature describing the role of miR-3470a is limited. A recent publication showed that miR-3470a promotes metastasis *in vivo*. [24] Overall, differentially expressed microRNAs overlapping

in all treatments appear to be involved in the regeneration of damaged cells and inhibition of apoptosis. Therefore, microRNAs may be involved in an adaptive response to drug-induced toxicity in order to repair drug-induced damage and prevent cell death. However, since most research regarding the function of these microRNAs was conducted in cancer investigation, these results may be biased. Future research should validate the function of these differentially expressed microRNAs in liver toxicity.

Apart from the microRNAs overlapping between the different treatments, several compound-specific changes on microRNA expression are found, which seems logical since the three compounds belong to different classes of hepatotoxicants. CsA is known to induce cholestasis in human liver, AM can induce microvesicular steatosis and APAP induces hepatocellular necrosis. [25-27] Pathway analysis of target genes of the differentially expressed microRNAs in HepG2 cells after CsA treatment reveals 2 significant pathways involving 6 of the 80 target genes. The significant pathways are involved in angiogenesis and the transcription factor HIF-2α, which are important in processes involved in tumor growth. Again, these results may be biased since important microRNA research is performed in cancer research. However, HIF-2α is also shown to be important in the regulation of lipid homeostasis in hepatocytes. [28] In chapter 4 we show that CsA treatment of HepG2 cells did not result in bile salt accumulation but increased levels of intracellular cholesterol. The results of this study indicate that microRNAs differentially expressed after CsA treatment may be involved in lipid homeostasis, which may play an important role in CsA-induced toxicity in HepG2 cells. The 10 target genes which show correlated expression to the differential microRNAs are mostly involved in cell cycle and proliferation, which is in concordance with the analysis of the microRNAs overlapping in all three treatments and pathway analysis results of the DEGs. Pathway analysis of the target genes of microRNAs differentially expressed after AM treatment resulted in 28 significant pathways, which were mostly involved in fatty acid homeostasis, transcription and translation. Since AM is known to induce steatosis in hepatocytes, these results suggest the involvement of microRNAs in fatty acid homeostasis and AM-induced toxicity. However, no target genes were differentially expressed after AM treatment. Pathway analysis of target genes of the differentially expressed microRNAs after APAP treatment did not result in significant pathways. Again, like in CsA treated cells, most correlated expressed target genes are involved in cell cycle and proliferation. In HepG2 cells, both pathway analysis of DEGs and target genes of differentially expressed microRNAs reveal changes on cell cycle processes in all treatments. Since HepG2 is a cancer cell line, these changes could be cell line-specific. Pathway analyses of target genes of the differentially expressed microRNAs in PMH after the three different treatments and investigation of correlated differential target genes did not result in a clear drug-specific response. Pathways were involved in multiple cellular processes, e.g. pathways involved in an inflammatory response and the circadian clock were significantly affected after treatment with AM and CsA.

Cytochrome P450 (CYP) enzymes are responsible for the metabolic activation of xenobiotics and their expression is regulated by post-transcriptional and post-translational mechanisms. MicroRNAs are believed to be involved in this post-transcriptional regulation. [29] APAP

metabolization is mainly catalyzed by CYP2E1 and Mohri et al. showed that miR-378 can repress the translation of CYP2E1 in human kidney cells. [30] APAP treatment did not affect the transcription of this microRNA in our HepG2 experiments; however, miR-378a and miR-378b and the Cyp2e1 mRNA are significantly down-regulated in PMH after CsA treatment. CsA and AM are mainly metabolized by CYP3A4, which can be regulated directly and indirectly by multiple microRNAs. MiR-148a was shown to indirectly regulate CYP3A4 transcription via the post-transcriptional regulation of the Pregnane X Receptor (PXR). [31] MiR-148a is significantly down-regulated after CsA and AM treatment in HepG2 cells, which could lead to increased levels of PXR and CYP3A4. However, CYP3A4 expression was not measured with the used microarrays and the levels of PXR mRNA are not changed after treatment with CsA and AM. Furthermore, CYP3A4 expression can be directly and indirectly regulated by miR-27b and mmu-miR-298. [32] These microRNAs can directly inhibit CYP3A4 translation or inhibit transcription of CYP3A4 via the post-transcriptional regulation of the Vitamin D Receptor (VDR). The expression of mmu-miR-298 was not measured with the used microarrays, but miR-27b is significantly down-regulated after CsA treatment in PMH. However, the expression of VDR mRNA was not assessed with the used microarrays and there is no direct homologue of CYP3A4 in mice. Overall, several differentially expressed microRNAs in HepG2 cells and PMH are shown to interact with genes involved in the metabolic activation of the used compounds. However, transcriptional regulation of their target genes is not observed in our experiments. Future research should investigate the possible interactions of microRNAs and CYP enzymes in human and mice.

Apart from the regulation of CYP3A4, miR-27b also targets genes involved in lipid metabolism. Vickers et al. showed the regulation of key genes in lipid metabolism, including PPARG, GPAM, and ANGPTL3. PPARG is involved in fatty acid storage and glucose metabolism and the mRNA expression is changed after CsA treatment in PMH with an FDR < 0.05, but the FC is only 1.22. The up-regulation of PPARG is in concordance with the down-regulated miR-27b. However, GPAM and ANGPTL3 are significantly down-regulated after CsA treatment (FC of -3.63 and -10.77, respectively), which suggest the presence of other regulators of these genes. Furthermore, decreased levels of miR-27b were found in plasma of rats with extrahepatic cholestasis after bile duct ligation. [5] Yamaura et al. suggest that decreased levels of miR-27b in plasma, together with decreased levels of several other microRNAs, including miR-99a, miR-185, and miR-361, can be used as biomarkers for cholestasis. Expression levels of these microRNAs are significantly down-regulated in PMH after treatment with CsA, which is known to induce cholestasis. MiR-99a and miR-361 are significantly up-regulated after treatment with AM and miR-185 is up-regulated in APAP exposed PMH. None of these microRNAs are differentially expressed in HepG2 cells. These microRNAs could possibly be used to distinguish cholestatic compounds from other hepatotoxic compounds, since these microRNAs were not significantly expressed in the other treatments or differentially expressed in the opposite direction.

Although concordant regulations of target genes are found for overexpressed microRNAs and down-regulated microRNAs, opposite reactions are also observed. Furthermore, multiple microRNAs are shown to target the same gene, but in most cases no regulation was seen on the mRNA level. Literature search revealed the possible involvement of microRNAs in processes underlying drug-induced hepatotoxicity and many of the described targets are not yet available in the miRTarBase lists. Targeted research of these microRNAs, genes and the proteins they code for, should be performed to investigate their role in drug-induced liver injury. Differentially expressed microRNAs in PMH could be backed-up with literature, where HepG2 data shows little to no concordance with the literature, which suggests that for microRNA-based investigation of liver toxicity, PMH is preferred over HepG2 cells. However, the significantly changed microRNAs in HepG2 may provide more information once complete elucidation of all target genes of microRNAs is achieved. Future research should validate the found differentially expressed microRNAs and their ability of altering mRNA expression and translation of their target genes.

Availability of supporting data

The data sets supporting the results of this article are included within the article and its additional files. Additional files are available upon request.

Table 1. *Total number of significantly expressed genes and microRNAs (False Discovery Rate < 0.05 and absolute Fold Change > 1.5) after treatment for 24 hours with the IC_{20} concentration of cyclosporin A, amiodarone and acetaminophen in HepG2 cells and primary mouse hepatocytes.*

	HepG2 cells			Primary Mouse Hepatocytes		
	CsA	*AM*	*APAP*	*CsA*	*AM*	*APAP*
mRNA	**3217**	**5**	**2273**	**3972**	**1458**	**1943**
Up-regulated	*1210*	*4*	*1199*	*1923*	*725*	*843*
Down-regulated	*2007*	*1*	*1074*	*2049*	*733*	*1100*
microRNA	**19**	**16**	**4**	**41**	**40**	**23**
Up-regulated	*4*	*7*	*2*	*17*	*27*	*15*
Down-regulated	*15*	*9*	*2*	*24*	*13*	*8*

Table 2.1. *Differentially expressed microRNAs in HepG2 cells after treatment for 24 hours with cyclosporin A, amiodarone and acetaminophen (FDR < 0.05 and absolute FC > 1.5).*

	CsA	AM	APAP
DOWN-REGULATED	hsa-miR-148a*	hsa-miR-125b-1*	hsa-miR-2110
	hsa-miR-202	hsa-miR-148a*	hsa-miR-3152-3p
	hsa-miR-2110	hsa-miR-2110	
	hsa-miR-3152-3p	hsa-miR-3140-3p	
	hsa-miR-3159	hsa-miR-3152-3p	
	hsa-miR-3168	hsa-miR-3689a-5p/hsa-miR-3689b/hsa-miR-3689e	
	hsa-miR-3612	hsa-miR-4296	
	hsa-miR-4296	hsa-miR-519c-3p	
	hsa-miR-519c-3p	hsa-miR-559	
	hsa-miR-525-3p		
	hsa-miR-559		
	hsa-miR-614		
	hsa-miR-888		
	hsa-miR-935		
	hsa-miRPlus-I152*		
UP-REGULATED	hsa-miR-1284	hsa-miR-1244	hsa-miR-1244
	hsa-miR-3939	hsa-miR-1284	hsa-miR-183
	hsa-miRPlus-C1076	hsa-miR-3178	
	hsv1-miR-H6-3p	hsa-miR-324-3p	
		hsa-miR-3656	
		hsa-miR-668	
		hsa-miRPlus-C1076	

Table 2.2. *Differentially expressed microRNAs in primary mouse hepatocytes after treatment for 24 hours with cyclosporin A, amiodarone and acetaminophen (FDR < 0.05 and absolute FC > 1.5).*

	CsA	AM	APAP
DOWN-REGULATED	mmu-miR-101c	mmu-miR-1934-3p	mmu-miR-1934-3p
	mmu-miR-106b-5p	mmu-miR-212-3p	mmu-miR-212-3p
	mmu-miR-130a-3p	mmu-miR-2861	mmu-miR-3057-5p
	mmu-miR-148b-3p	mmu-miR-3057-5p	mmu-miR-6368
	mmu-miR-152-3p	mmu-miR-3098-5p	mmu-miR-680
	mmu-miR-183-5p	mmu-miR-3102-5p	mmu-miR-710
	mmu-miR-1839-5p	mmu-miR-3102-5p.2-5p	mmu-miR-711
	mmu-miR-185-5p	mmu-miR-6368	mmu-miR-721
	mmu-miR-193a-3p	mmu-miR-6401	
	mmu-miR-203-3p	mmu-miR-680	
	mmu-miR-212-3p	mmu-miR-710	
	mmu-miR-21a-5p	mmu-miR-711	
	mmu-miR-221-3p	mmu-miR-877-5p	
	mmu-miR-27b-3p		
	mmu-miR-30a-3p		
	mmu-miR-30e-5p		
	mmu-miR-361-5p		
	mmu-miR-374c-5p		
	mmu-miR-378a-3p		
	mmu-miR-378b		
	mmu-miR-425-5p		
	mmu-miR-5097		
	mmu-miR-877-5p		
	mmu-miR-99a-5p		
UP-REGULATED	mmu-miR-1306-3p	mmu-miR-128-3p	mmu-miR-151-5p
	mmu-miR-1892	mmu-miR-140-5p	mmu-miR-1839-5p
	mmu-miR-3095-3p	mmu-miR-151-5p	mmu-miR-185-5p
	mmu-miR-3096b-3p	mmu-miR-183-5p	mmu-miR-202-3p
	mmu-miR-3470a	mmu-miR-1839-5p	mmu-miR-21a-3p
	mmu-miR-3473a	mmu-miR-202-3p	mmu-miR-22-5p
	mmu-miR-3473b	mmu-miR-20a-5p	mmu-miR-3058-5p
	mmu-miR-370-3p	mmu-miR-20b-5p	mmu-miR-3096a-5p
	mmu-miR-494-3p	mmu-miR-21a-3p	mmu-miR-30a-3p
	mmu-miR-5105	mmu-miR-21a-5p	mmu-miR-30c-2-3p
	mmu-miR-5119	mmu-miR-25-3p	mmu-miR-30e-3p
	mmu-miR-5121	mmu-miR-28a-5p	mmu-miR-3470a
	mmu-miR-5622-3p	mmu-miR-3058-5p	mmu-miR-500-3p
	mmu-miR-6243	mmu-miR-3096a-5p	mmu-miR-652-3p
	mmu-miR-6360	mmu-miR-30c-2-3p	mmu-miR-697
	mmu-miR-6378	mmu-miR-30e-3p	
	mmu-miR-712-5p	mmu-miR-3470a	
		mmu-miR-361-5p	
		mmu-miR-374c-5p	
		mmu-miR-378b	
		mmu-miR-423-5p	
		mmu-miR-425-5p	
		mmu-miR-500-3p	
		mmu-miR-652-3p	
		mmu-miR-98-5p	
		mmu-miR-99a-5p	
		mmu-miR-99b-5p	

Table 3. *Results of the pathway over-representation analysis of the target genes of differentially expressed microRNAs after treatment of HepG2 cells for 24 hours with cyclosporin A, amiodarone and acetaminophen.*

Pathway name	Pathway size	Overlap input genes	% overlap	p-value	q-value	Pathway source
Cyclosporin A						
Angiogenesis	19	3	15,80%	0,00	0,05	Wikipathways
HIF-2-alpha transcription factor network	35	4	11,40%	0,00	0,03	PID
Amiodarone						
Fatty Acyl-CoA Biosynthesis	4	4	100,00%	0,00	0,00	PID
Metabolism of polyamines	4	3	75,00%	0,00	0,00	PID
Spermidine and Spermine Biosynthesis	6	4	66,70%	0,00	0,00	SMPDB
Telomere Maintenance	10	3	30,00%	0,00	0,01	Wikipathways
Non-homologous end-joining - Homo sapiens (human)	13	3	23,10%	0,00	0,03	KEGG
Metabolism of polyamines	14	3	21,40%	0,00	0,04	Reactome
Early Phase of HIV Life Cycle	14	3	21,40%	0,00	0,04	Reactome
Fatty Acyl-CoA Biosynthesis	20	4	20,00%	0,00	0,01	Reactome
hypoxia and p53 in the cardiovascular system	22	4	18,20%	0,00	0,01	PID
hypoxia and p53 in the cardiovascular system	22	4	18,20%	0,00	0,01	BioCarta
Extension of Telomeres	28	4	14,30%	0,00	0,03	Reactome
Eukaryotic Translation Initiation	54	7	13,00%	0,00	0,00	PID
Peptide chain elongation	94	12	12,80%	0,00	0,00	PID
Peptide chain elongation	95	12	12,60%	0,00	0,00	Reactome
Validated targets of C-MYC transcriptional activation	87	11	12,60%	0,00	0,00	PID
Eukaryotic Translation Elongation	100	12	12,00%	0,00	0,00	Reactome
mechanism of gene regulation by peroxisome proliferators via ppara	52	6	11,50%	0,00	0,01	BioCarta
mechanism of gene regulation by peroxisome proliferators via ppara	52	6	11,50%	0,00	0,01	PID
Cytoplasmic Ribosomal Proteins	88	10	11,40%	0,00	0,00	Wikipathways
Transcriptional Regulation of White Adipocyte Differentiation	44	5	11,40%	0,00	0,02	Reactome
Eukaryotic Translation Termination	93	10	10,80%	0,00	0,00	PID
Viral mRNA Translation	93	10	10,80%	0,00	0,00	PID
Eukaryotic Translation Termination	94	10	10,60%	0,00	0,00	Reactome
Formation of a pool of free 40S subunits	105	11	10,50%	0,00	0,00	PID
Formation of a pool of free 40S subunits	106	11	10,40%	0,00	0,00	Reactome
Nonsense Mediated Decay Independent of the Exon Junction Complex	99	10	10,10%	0,00	0,00	Reactome
Nonsense Mediated Decay Enhanced by the Exon Junction Complex	110	11	10,00%	0,00	0,00	Reactome
Nonsense-Mediated Decay	110	11	10,00%	0,00	0,00	Reactome
Acetaminophen						
No significant pathways were found						

Table 4. *Results of the pathway over-representation analysis of the target genes of differentially expressed microRNAs after treatment of primary mouse hepatocytes for 24 hours with cyclosporin A, amiodarone and acetaminophen.*

Pathway name	Pathway size	Overlap input genes	% overlap	p-value	q-value	Pathway source
Cyclosporin A						
Formation of editosomes by ADAR proteins	2	2	100,00%	0,00	0,02	Reactome
C6 deamination of adenosine	2	2	100,00%	0,00	0,02	Reactom
mRNA Editing: A to I Conversion	2	2	100,00%	0,00	0,02	Reactome
BH3-only proteins associate with and inactivate anti-apoptotic BCL-2 members	7	3	42,90%	0,00	0,02	Reactome
MASTL Facilitates Mitotic Progression	10	4	40,00%	0,00	0,01	Reactome
Activation of BH3-only proteins	19	6	31,60%	0,00	0,00	Reactome
Circadian Clock	13	4	30,80%	0,00	0,01	Reactome
Circadian Clock	13	4	30,80%	0,00	0,01	Reactome
Interleukin-6 signaling	10	3	30,00%	0,01	0,04	Reactome
Oxidative Damage	17	5	29,40%	0,00	0,01	Wikipathways
SHC-related events triggered by IGF1R	18	5	27,80%	0,00	0,01	Reactome
ERK/MAPK targets	20	5	25,00%	0,00	0,01	Reactome
Signaling by BMP	21	5	23,80%	0,00	0,01	Reactome
Signaling of Hepatocyte Growth Factor Receptor	34	8	23,50%	0,00	0,00	Wikipathways
Beta-catenin phosphorylation cascade	17	4	23,50%	0,00	0,03	Reactome
Hedgehog Signaling Pathway	22	5	22,70%	0,00	0,01	Wikipathways
Trafficking of AMPA receptors	27	6	22,20%	0,00	0,01	Reactome
Glutamate Binding, Activation of AMPA Receptors and Synaptic Plasticity	27	6	22,20%	0,00	0,01	Reactome
Frs2-mediated activation	18	4	22,20%	0,00	0,03	Reactome
Nuclear Events (kinase and transcription factor activation)	23	5	21,70%	0,00	0,02	Reactome
Tie2 Signaling	19	4	21,10%	0,00	0,04	Reactome
MAPK targets/ Nuclear events mediated by MAP kinases	29	6	20,70%	0,00	0,01	Reactome
Intrinsic Pathway for Apoptosis	39	8	20,50%	0,00	0,00	Reactome
Degradation of beta-catenin by the destruction complex	25	5	20,00%	0,00	0,02	Reactome
Prolonged ERK activation events	20	4	20,00%	0,01	0,04	Reactome
Long-term potentiation - Mus musculus (mouse)	66	13	19,70%	0,00	0,00	KEGG
Endometrial cancer - Mus musculus (mouse)	52	10	19,20%	0,00	0,00	KEGG
Colorectal cancer - Mus musculus (mouse)	64	12	18,80%	0,00	0,00	KEGG
Amino acid transport across the plasma membrane	32	6	18,80%	0,00	0,01	Reactome
Chronic myeloid leukemia - Mus musculus (mouse)	74	13	17,60%	0,00	0,00	KEGG
p38 MAPK Signaling Pathway	34	6	17,60%	0,00	0,02	Wikipathways

Table 4. *continuation*

Pathway name	Pathway size	Overlap input genes	% overlap	p-value	q-value	Pathway source
Cyclosporin A						
Signal transduction by L1	*34*	*6*	*17,60%*	*0,00*	*0,02*	*Reactome*
Acute myeloid leukemia - Mus musculus (mouse)	*57*	*10*	*17,50%*	*0,00*	*0,00*	*KEGG*
Signalling to ERKs	*35*	*6*	*17,10%*	*0,00*	*0,02*	*Reactome*
Apoptosis	*83*	*14*	*16,90%*	*0,00*	*0,00*	*Wikipathways*
Hedgehog signaling pathway - Mus musculus (mouse)	*49*	*8*	*16,30%*	*0,00*	*0,01*	*KEGG*
Renal cell carcinoma - Mus musculus (mouse)	*68*	*11*	*16,20%*	*0,00*	*0,00*	*KEGG*
Morphine addiction - Mus musculus (mouse)	*93*	*15*	*16,10%*	*0,00*	*0,00*	*KEGG*
Circadian rhythm - Mus musculus (mouse)	*31*	*5*	*16,10%*	*0,01*	*0,04*	*KEGG*
IL-7 Signaling Pathway	*44*	*7*	*15,90%*	*0,00*	*0,01*	*Wikipathways*
Amyotrophic lateral sclerosis (ALS) - Mus musculus (mouse)	*52*	*8*	*15,40%*	*0,00*	*0,01*	*KEGG*
TGF Beta Signaling Pathway	*52*	*8*	*15,40%*	*0,00*	*0,01*	*Wikipathways*
Glioma - Mus musculus (mouse)	*66*	*10*	*15,20%*	*0,00*	*0,00*	*KEGG*
VEGF signaling pathway - Mus musculus (mouse)	*60*	*9*	*15,00%*	*0,00*	*0,01*	*KEGG*
Pancreatic cancer - Mus musculus (mouse)	*67*	*10*	*14,90%*	*0,00*	*0,01*	*KEGG*
Heart Development	*47*	*7*	*14,90%*	*0,00*	*0,02*	*Wikipathways*
Long-term depression - Mus musculus (mouse)	*61*	*9*	*14,80%*	*0,00*	*0,01*	*KEGG*
NCAM signaling for neurite out-growth	*55*	*8*	*14,80%*	*0,00*	*0,01*	*Reactome*
MAP kinase activation in TLR cascade	*48*	*7*	*14,60%*	*0,00*	*0,02*	*Reactome*
Splicing factor NOVA regulated synpatic proteins	*41*	*6*	*14,60%*	*0,00*	*0,03*	*Wikipathways*
mTOR signaling pathway - Mus musculus (mouse)	*62*	*9*	*14,50%*	*0,00*	*0,01*	*KEGG*
GABAergic synapse - Mus musculus (mouse)	*90*	*13*	*14,40%*	*0,00*	*0,00*	*KEGG*
Non-small cell lung cancer - Mus musculus (mouse)	*57*	*8*	*14,00%*	*0,00*	*0,01*	*KEGG*
Amino acid and oligopeptide SLC transporters	*50*	*7*	*14,00%*	*0,00*	*0,02*	*Reactome*
Neurotrophin signaling pathway - Mus musculus (mouse)	*123*	*17*	*13,80%*	*0,00*	*0,00*	*KEGG*
Wnt Signaling Pathway NetPath	*109*	*15*	*13,80%*	*0,00*	*0,00*	*Wikipathways*
Retrograde endocannabinoid signaling - Mus musculus (mouse)	*104*	*14*	*13,50%*	*0,00*	*0,00*	*KEGG*

Table 4. *continuation*

Pathway name	Pathway size	Overlap input genes	% overlap	p-value	q-value	Pathway source
Cyclosporin A						
Kit Receptor Signaling Pathway	*67*	*9*	*13,40%*	*0,00*	*0,01*	*Wikipathways*
TGF-beta Receptor Signaling Pathway	*150*	*20*	*13,30%*	*0,00*	*0,00*	*Wikipathways*
Dopaminergic synapse - Mus musculus (mouse)	*135*	*18*	*13,30%*	*0,00*	*0,00*	*KEGG*
Glutamatergic synapse - Mus musculus (mouse)	*115*	*15*	*13,00%*	*0,00*	*0,00*	*KEGG*
IL-3 Signaling Pathway	*100*	*13*	*13,00%*	*0,00*	*0,00*	*Wikipathways*
MyD88 cascade initiated on plasma membrane	*69*	*9*	*13,00%*	*0,00*	*0,01*	*Reactome*
Toll Like Receptor 10 (TLR10) Cascade	*69*	*9*	*13,00%*	*0,00*	*0,01*	*Reactome*
Toll Like Receptor 5 (TLR5) Cascade	*69*	*9*	*13,00%*	*0,00*	*0,01*	*Reactome*
MyD88:Mal cascade initiated on plasma membrane	*69*	*9*	*13,00%*	*0,00*	*0,01*	*Reactome*
Toll Like Receptor TLR1:TLR2 Cascade	*69*	*9*	*13,00%*	*0,00*	*0,01*	*Reactome*
MAPK signaling pathway	*158*	*20*	*12,70%*	*0,00*	*0,00*	*Wikipathways*
ErbB signaling pathway - Mus musculus (mouse)	*87*	*11*	*12,60%*	*0,00*	*0,01*	*KEGG*
EGFR1 Signaling Pathway	*176*	*22*	*12,50%*	*0,00*	*0,00*	*Wikipathways*
Prostate cancer - Mus musculus (mouse)	*89*	*11*	*12,40%*	*0,00*	*0,01*	*KEGG*
Toll Like Receptor TLR6:TLR2 Cascade	*73*	*9*	*12,30%*	*0,00*	*0,02*	*Reactome*
Toll Like Receptor 2 (TLR2) Cascade	*73*	*9*	*12,30%*	*0,00*	*0,02*	*Reactome*
TRAF6 mediated induction of NFkB and MAP kinases upon TLR7/8 or 9 activation	*73*	*9*	*12,30%*	*0,00*	*0,02*	*Reactome*
Hepatitis B - Mus musculus (mouse)	*148*	*18*	*12,20%*	*0,00*	*0,00*	*KEGG*
TGF-beta signaling pathway - Mus musculus (mouse)	*82*	*10*	*12,20%*	*0,00*	*0,01*	*KEGG*
Apoptosis - Mus musculus (mouse)	*82*	*10*	*12,20%*	*0,00*	*0,01*	*KEGG*
Circadian entrainment - Mus musculus (mouse)	*99*	*12*	*12,10%*	*0,00*	*0,01*	*KEGG*
Estrogen signaling pathway - Mus musculus (mouse)	*99*	*12*	*12,10%*	*0,00*	*0,01*	*KEGG*
Adherens junction - Mus musculus (mouse)	*75*	*9*	*12,00%*	*0,00*	*0,02*	*KEGG*
MyD88 dependent cascade initiated on endosome	*75*	*9*	*12,00%*	*0,00*	*0,02*	*Reactome*
Toll Like Receptor 7/8 (TLR7/8) Cascade	*75*	*9*	*12,00%*	*0,00*	*0,02*	*Reactome*
ESC Pluripotency Pathways	*110*	*13*	*11,80%*	*0,00*	*0,01*	*Wikipathways*
IL-5 Signaling Pathway	*68*	*8*	*11,80%*	*0,00*	*0,03*	*Wikipathways*
Amphetamine addiction - Mus musculus (mouse)	*68*	*8*	*11,80%*	*0,00*	*0,03*	*KEGG*
Axon guidance	*225*	*26*	*11,60%*	*0,00*	*0,00*	*Reactome*
Activated TLR4 signalling	*95*	*11*	*11,60%*	*0,00*	*0,01*	*Reactome*
Oocyte meiosis - Mus musculus (mouse)	*113*	*13*	*11,50%*	*0,00*	*0,01*	*KEGG*

Table 4. *continuation*

Pathway name	Pathway size	Overlap input genes	% overlap	p-value	q-value	Pathway source
Cyclosporin A						
Chagas disease (American trypanosomiasis) - Mus musculus (mouse)	104	12	11,50%	0,00	0,01	KEGG
Gap junction - Mus musculus (mouse)	88	10	11,40%	0,00	0,02	KEGG
Toll Like Receptor 9 (TLR9) Cascade	79	9	11,40%	0,00	0,02	Reactome
Progesterone-mediated oocyte maturation - Mus musculus (mouse)	89	10	11,20%	0,00	0,02	KEGG
Wnt signaling pathway - Mus musculus (mouse)	144	16	11,10%	0,00	0,00	KEGG
Melanoma - Mus musculus (mouse)	73	8	11,00%	0,01	0,04	KEGG
NGF signalling via TRKA from the plasma membrane	184	20	10,90%	0,00	0,00	Reactome
Axon guidance - Mus musculus (mouse)	129	14	10,90%	0,00	0,01	KEGG
Apoptosis	101	11	10,90%	0,00	0,02	Reactome
Developmental Biology	307	33	10,80%	0,00	0,00	Reactome
Androgen Receptor Signaling Pathway	111	12	10,80%	0,00	0,01	Wikipathways
Spinal Cord Injury	103	11	10,80%	0,00	0,02	Wikipathways
Gastric acid secretion - Mus musculus (mouse)	74	8	10,80%	0,01	0,05	KEGG
B cell receptor signaling pathway - Mus musculus (mouse)	74	8	10,80%	0,01	0,05	KEGG
Calcium Regulation in the Cardiac Cell	150	16	10,70%	0,00	0,00	Wikipathways
Neurotransmitter Receptor Binding And Downstream Transmission In The Postsynaptic Cell	133	14	10,60%	0,00	0,01	Reactome
HIF-1 signaling pathway - Mus musculus (mouse)	113	12	10,60%	0,00	0,01	KEGG
Proteoglycans in cancer - Mus musculus (mouse)	229	24	10,50%	0,00	0,00	KEGG
Toll Like Receptor 4 (TLR4) Cascade	106	11	10,40%	0,00	0,02	Reactome
Transmission across Chemical Synapses	178	18	10,20%	0,00	0,00	Reactome
Senescence and Autophagy	98	10	10,20%	0,00	0,03	Wikipathways
Insulin secretion - Mus musculus (mouse)	88	9	10,20%	0,01	0,04	KEGG
MAPK signaling pathway - Mus musculus (mouse)	257	26	10,10%	0,00	0,00	KEGG
IL-6 signaling Pathway	99	10	10,10%	0,00	0,03	Wikipathways
TRIF-mediated TLR3/TLR4 signaling	89	9	10,10%	0,01	0,04	Reactome
MyD88-independent cascade	89	9	10,10%	0,01	0,04	Reactome
Toll Like Receptor 3 (TLR3) Cascade	89	9	10,10%	0,01	0,04	Reactome

Table 4. *continuation*

Pathway name	Pathway size	Overlap input genes	% overlap	p-value	q-value	Pathway source
Amiodarone						
MASTL Facilitates Mitotic Progression	10	3	30,00%	0,00	0,02	Reactome
Interleukin-6 signaling	10	3	30,00%	0,00	0,02	Reactome
Signaling of Hepatocyte Growth Factor Receptor	34	8	23,50%	0,00	0,00	Wikipathways
Oxidative Damage	17	4	23,50%	0,00	0,01	Wikipathways
Circadian Clock	13	3	23,10%	0,00	0,04	Reactome
Circadian Clock	13	3	23,10%	0,00	0,04	Reactome
Spry regulation of FGF signaling	15	3	20,00%	0,00	0,04	Reactome
Trafficking of GluR2-containing AMPA receptors	15	3	20,00%	0,00	0,04	Reactome
GRB2:SOS provides linkage to MAPK signaling for Intergrins	15	3	20,00%	0,00	0,04	Reactome
Rap1 signalling	15	3	20,00%	0,00	0,04	Reactome
Hedgehog Signaling Pathway	22	4	18,20%	0,00	0,02	Wikipathways
Trafficking of AMPA receptors	27	4	14,80%	0,00	0,04	Reactome
Glutamate Binding, Activation of AMPA Receptors and Synaptic Plasticity	27	4	14,80%	0,00	0,04	Reactome
EGFR downregulation	27	4	14,80%	0,00	0,04	Reactome
Hedgehog signaling pathway - Mus musculus (mouse)	49	7	14,30%	0,00	0,00	KEGG
Signalling to ERKs	35	5	14,30%	0,00	0,02	Reactome
IL-7 Signaling Pathway	44	6	13,60%	0,00	0,01	Wikipathways
Renal cell carcinoma - Mus musculus (mouse)	68	9	13,20%	0,00	0,00	KEGG
mTOR signaling pathway - Mus musculus (mouse)	62	8	12,90%	0,00	0,00	KEGG
Intrinsic Pathway for Apoptosis	39	5	12,80%	0,00	0,03	Reactome
Colorectal cancer - Mus musculus (mouse)	64	8	12,50%	0,00	0,00	KEGG
Nicotine addiction - Mus musculus (mouse)	40	5	12,50%	0,00	0,03	KEGG
Amino acid transport across the plasma membrane	32	4	12,50%	0,01	0,05	Reactome
IL-3 Signaling Pathway	100	12	12,00%	0,00	0,00	Wikipathways
Pancreatic cancer - Mus musculus (mouse)	67	8	11,90%	0,00	0,01	KEGG
VEGF signaling pathway - Mus musculus (mouse)	60	7	11,70%	0,00	0,01	KEGG
Amyotrophic lateral sclerosis (ALS) - Mus musculus (mouse)	52	6	11,50%	0,00	0,02	KEGG
Endometrial cancer - Mus musculus (mouse)	52	6	11,50%	0,00	0,02	KEGG

Table 4. *continuation*

Pathway name	Pathway size	Overlap input genes	% overlap	p-value	q-value	Pathway source
Amiodarone						
TGF Beta Signaling Pathway	*52*	*6*	*11,50%*	*0,00*	*0,02*	*Wikipathways*
Endochondral Ossification	*62*	*7*	*11,30%*	*0,00*	*0,01*	*Wikipathways*
GABAergic synapse - Mus musculus (mouse)	*90*	*10*	*11,10%*	*0,00*	*0,00*	*KEGG*
Morphine addiction - Mus musculus (mouse)	*93*	*10*	*10,80%*	*0,00*	*0,00*	*KEGG*
Chronic myeloid leukemia - Mus musculus (mouse)	*74*	*8*	*10,80%*	*0,00*	*0,01*	*KEGG*
IL-2 Signaling Pathway	*76*	*8*	*10,50%*	*0,00*	*0,01*	*Wikipathways*
Kit Receptor Signaling Pathway	*67*	*7*	*10,40%*	*0,00*	*0,02*	*Wikipathways*
IL-5 Signaling Pathway	*68*	*7*	*10,30%*	*0,00*	*0,02*	*Wikipathways*
Wnt Signaling Pathway NetPath	*109*	*11*	*10,10%*	*0,00*	*0,00*	*Wikipathways*
TGF-beta Receptor Signaling Pathway	*150*	*15*	*10,00%*	*0,00*	*0,00*	*Wikipathways*
Amino acid and oligopeptide SLC transporters	*50*	*5*	*10,00%*	*0,01*	*0,05*	*Reactome*
Acetaminophen						
No significant pathways were found						

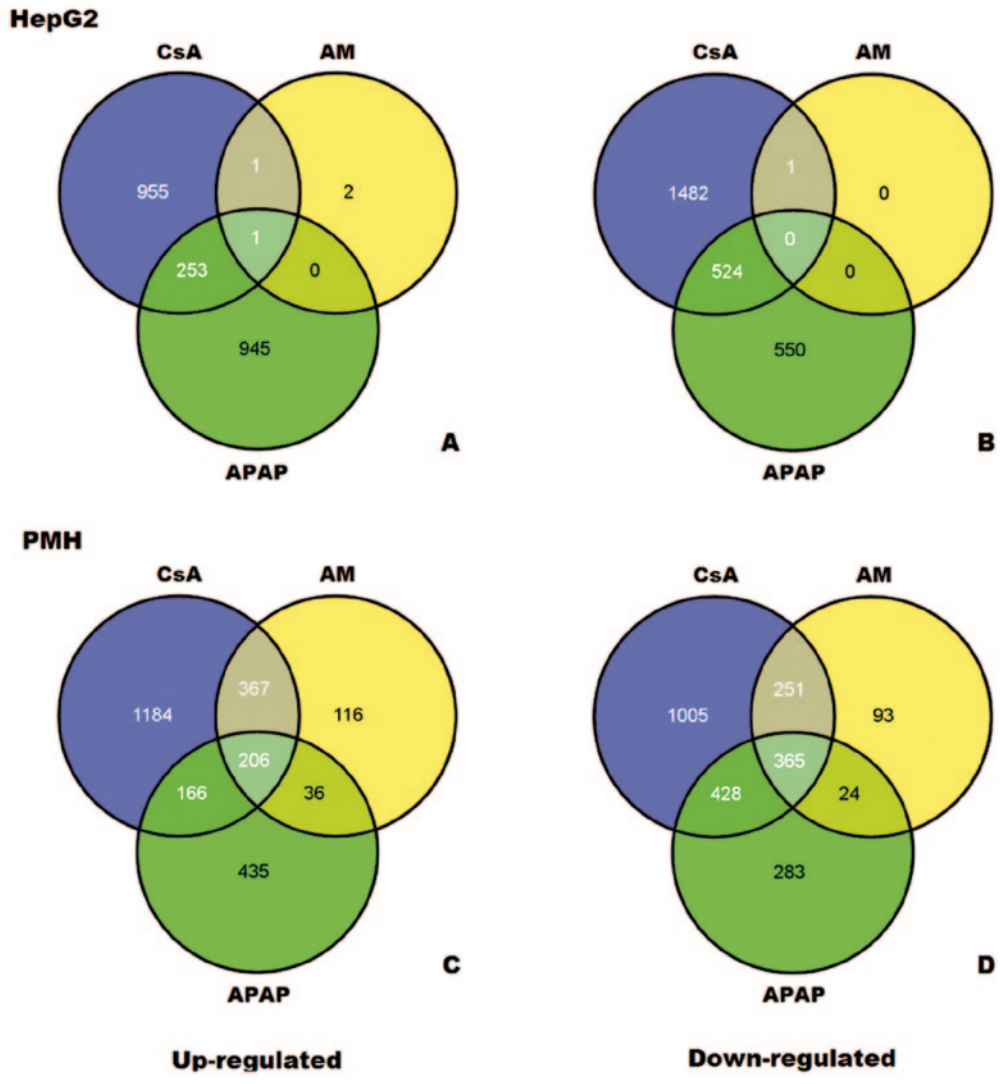

Figure 1. *Overlap of up-regulated and down-regulated differentially expressed genes in HepG2 cells (A and B, respectively) and primary mouse hepatocytes (C and D, respectively) after treatment with cyclosporin A, amiodarone and acetaminophen.*

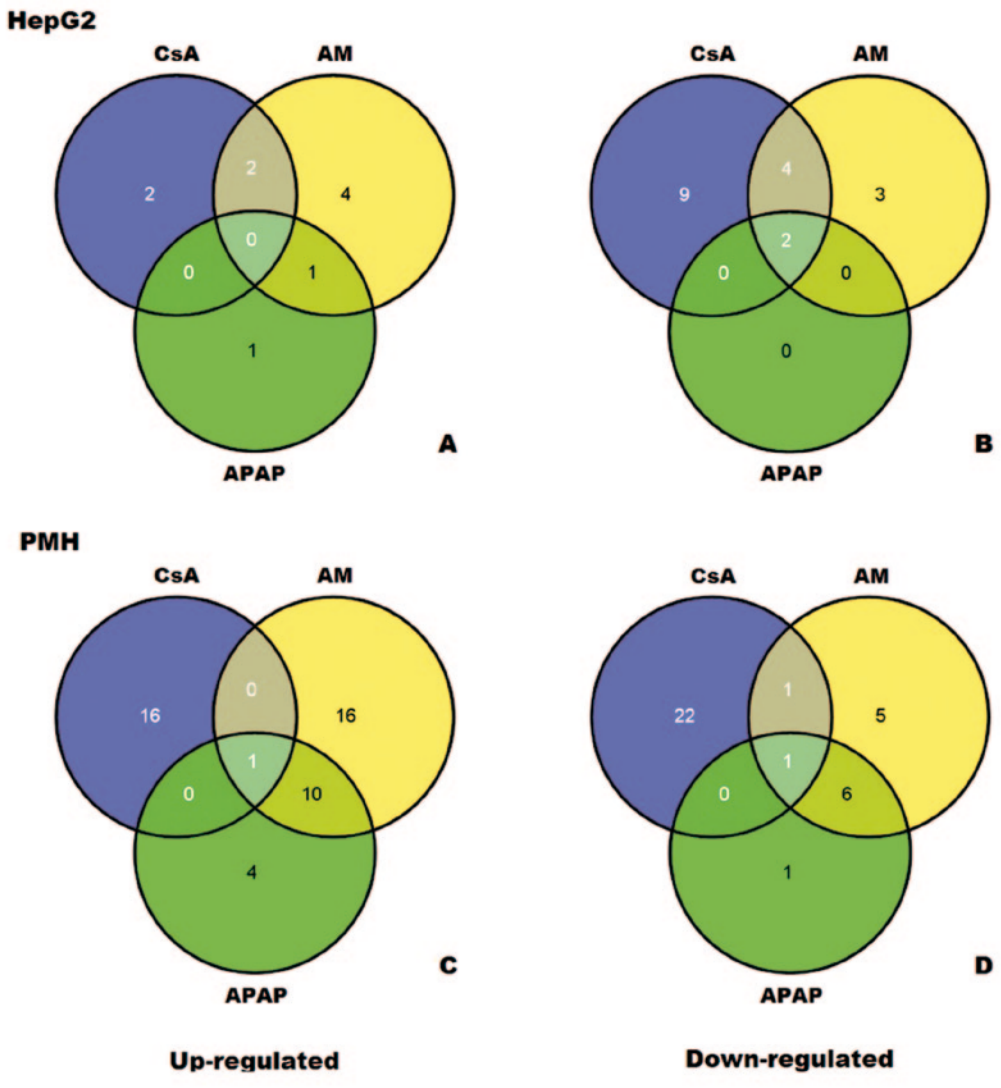

Figure 2. *Overlap of up-regulated and down-regulated differentially expressed microRNAs in HepG2 cells (A and B, respectively) and primary mouse hepatocytes (C and D, respectively) after treatment with cyclosporin A, amiodarone and acetaminophen.*

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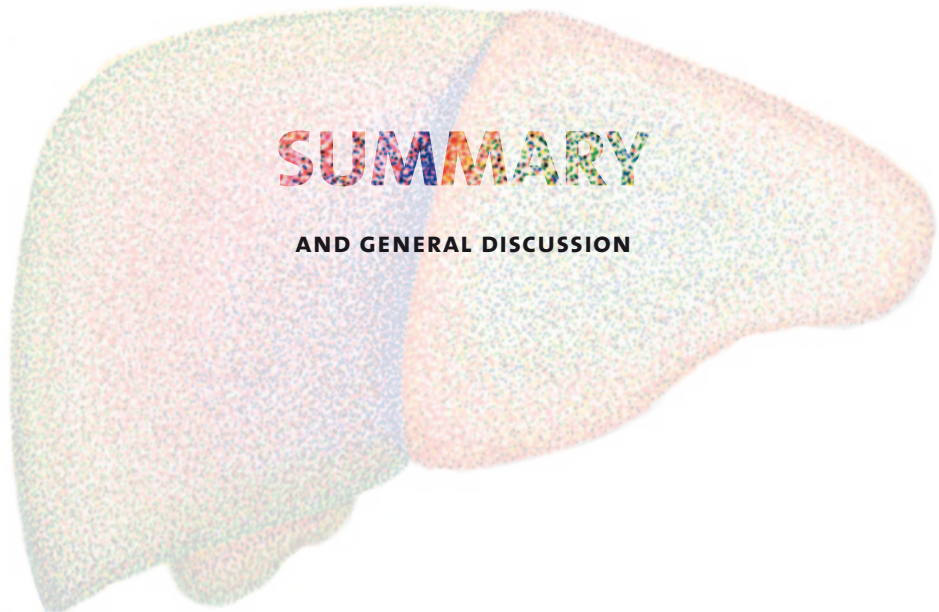
Chapter 7



Van den Hof, W.F.P.M.

The research described in this thesis, was performed in order to investigate the feasibility of using *in vitro* liver models to classify hepatotoxicants and to further unravel the mechanisms underlying drug-induced liver injury. Current toxicity screening is dependent on animal experiments; however, despite being assessed as safe based on the outcome of such animal experimentation, many toxic drug-candidates still progress into clinical trials and even onto the market. [1] While the liver is the most important organ in drug metabolization and excretion, it is vulnerable to drug-induced toxicity. There is a need for reliable *in vitro* high throughput toxicity screenings, in particular since the number of drug candidates increase every year. Different classes of hepatotoxicity are considered, including necrosis, steatosis and cholestasis. These classes have a different phenotypical profile. Necrosis is a form of premature cell death, steatosis is characterized by the accumulation of lipids inside the cell and accumulation of bile constituents, like bile salts, is characteristic for cholestasis. [2-4] For most of the traditional biomarkers for liver injury, the differentiation between different classes of hepatotoxicity is difficult. Therefore, great effort is put in developing new screening methods in order to find novel and more accurate biomarkers. In the field of toxicogenomics different platforms for generating molecular information are considered. The genome-wide profiling of RNA molecules, e.g. messenger RNA and microRNA, is better known as transcriptomics and represents the most used platform in toxicogenomics research. Proteomics is used to investigate the global translation of mRNA into proteins and post-translational modifications. Metabonomics investigates the level of metabolites. All these platforms individually provide valuable information about cellular reactions to compound exposure; however, integrative analysis of all platforms has the potential of fully elucidating the mechanisms underlying drug-induced liver injury. In order to investigate drug-induced liver injury *in vitro*, multiple models are proposed as relevant alternative to the *in vivo* tests in laboratory animals. Liver slices keep a three-dimensional configuration and contain all cell types and functions of the intact liver. [5] However, preparation and culturing of liver slices is laborious and long-term exposures are impossible. Primary human hepatocytes are considered to be one of the best *in vitro* liver models, while these cells show largely the same behavior as hepatocytes *in vivo*. [6] However, human liver donors are scarce and due to interindividual variation, the comparison of multiple experiments is difficult. Stem cells may provide an unlimited supply of primary hepatocytes and therefore possibly present a valuable alternative model. Up till now, difficulties with differentiation protocols and culturing hinder the use of stem cell-derived hepatocytes in toxicity screenings.

Investigating hepatotoxicity using transcriptomics

A different liver model worth exploring is presented by immortalized cell lines of which HepG2 and HepaRG cells are used most frequently. Both represent a human liver cell model and are easy and cheap to culture. HepG2 cells show lower baseline levels of phase I and phase II enzymes than HepaRG, but the response towards hepatotoxicants is comparable. [7] In **Chapter 2** we therefore aim to prove the principal that HepG2 cells can be used to classify known hepatotoxicants and non-hepatotoxicants based on their distinctive gene expression profiles. For classification we use Prediction Analysis for Microarrays. Using 36 genes, we are able to classify hepatotoxicants and non-hepatotoxicants with an accuracy of 92% for the training set and 91% for the validation set. Furthermore, a second model is set-up in an attempt to sub-classify cholestatic compounds. This sub-classification of cholestatic compounds results in an accurate prediction of 8 out of 9 cholestatic compounds with an overall accuracy of 93%, using 12 genes. The genes that are selected for the classification of hepatotoxicity and cholestasis, indicate the possible involvement of endoplasmic reticulum stress and the unfolded protein response in drug-induced liver injury. Although we are able to accurately classify hepatotoxicants using transcriptomic analyses of HepG2 cells, the number of compounds should be increased to improve and validate this prediction method.

Drug-induced cholestasis has a high mortality rate and is one of the most severe manifestations of drug-induced hepatotoxicity. Inhibition of transport proteins resulting in intracellular bile accumulation has been suggested as the underlying mechanism. We therefore aim to benchmark data from three *in vitro* liver models for the investigation of drug-induced cholestasis against data from cholestatic patients, as described in **Chapter 3**. Microarray data of HepG2 cells, primary mouse hepatocytes and primary human hepatocytes exposed to three cholestatic compounds, namely cyclosporin A, chlorpromazine and ethinyl estradiol, are compared to transcriptomic data of liver samples from cholestatic patients. Overlapping differentially expressed genes in all treatments are investigated to find an *in vitro* fingerprint of drug-induced cholestasis. Only a limited number of significantly affected genes appear to overlap in all three treatments in primary mouse and human hepatocytes, and none of these are differentially expressed in the *in vivo* samples. Treatment of HepG2 cells with these three compounds, though, results in 151 overlapping differentially expressed genes. 13 of these genes appear also differentially expressed in the *in vivo* cholestasis samples, and these point towards the involvement of the unfolded protein response in cholestasis. This is in concordance with the results presented in **Chapter 2**, which suggest that endoplasmic reticulum stress and the unfolded protein response may play an important role in hepatotoxicity, and especially in cholestasis. The limited overlap of differentially expressed genes after treatment with these three cholestatic compounds indicates that different mechanisms are involved in the induced toxicity. A previously published pathway for drug-induced cholestasis is then used to visualize the gene expression changes in human *in vivo* cholestasis. Genes involved in the uptake and synthesis of bile salts are down-regulated and the transcription of hepatocellular export proteins is up-regulated, thus indicating an adaptive response of the diseased liver in order to reduce intracellular bile salt levels and to prevent further bile salt accumulation. Indications of an adaptive response are also observed in the *in vitro* models after treatment with the cholestatic compounds. Furthermore, drug-specific changes on expression levels of genes in the cholestasis pathway are observed that may result in intracellular accumulation of bile salts.

Multi-omics analysis in hepatotoxicity

In order to further unravel the mechanisms underlying drug-induced toxicity, a multi-omics approach is performed in **Chapter 4**. HepG2 cells are treated with the cholestatic compound cyclosporin A, and mRNA, microRNA, and metabonomic profiling is performed. Treatment of HepG2 cells diminish intracellular bile salt levels and induce an intracellular accumulation of cholesterol. Pathway analysis is performed using the differentially expressed genes and significantly affected metabolites. An integrated pathway analysis results in more relevant pathways than the separate analyses of the two datasets. Integrated pathway analysis indicates the significant change of pathways involved in cell cycle and cellular metabolism. Furthermore, significant changes are found for pathways involved in protein processing of the endoplasmic reticulum, bile acid biosynthesis and cholesterol metabolism. Moreover, the pathway for drug-induced cholestasis described in **Chapter 3**, is supplemented with possible interacting microRNAs. Analysis of the genes, microRNAs and metabolites of this pathway provides valuable information about cholestatic properties of compounds and upon proper validation, may therefore be used to screen new drug candidates for their possible cholestasis-inducing potential. The findings in this chapter confirm that an integrated omics approach, combining transcriptomic and metabonomic analyses of HepG2 cells, facilitates further unravelling of mechanisms underlying cyclosporin A-induced hepatotoxicity.

However, multiple important genes are minimally expressed in HepG2 cells, including genes involved in bile acid synthesis and important export proteins including the Bile Salt Export Pump. Up till now, toxicity screenings are mostly performed on rodents, which would suggest that primary hepatocytes from rodents present a relevant *in vitro* liver model. Previous research indicates that primary mouse hepatocytes are metabolically more stable than primary rat hepatocytes. [8] Primary mouse hepatocytes are therefore used in the investigations described in **Chapter 5**, to further elucidate the mechanisms underlying cyclosporin A-induced toxicity. After treatment with cyclosporin A, expression levels of proteins, mRNAs, microRNAs and metabolites are analyzed. Integrated analyses of significantly affected proteins and differentially expressed genes reveals that protein disulfide isomerase family A, member 4 (Pdia4) is significantly up-regulated on both the protein level and mRNA level. MicroRNAs can affect the stability en translation of target mRNAs and several databases exist with experimentally validated and predicted targets of microRNAs. [9] In this study, no experimental validated microRNAs are identified that target the Pdia4 mRNA. Mmu-miR-182-5p is predicted by two separate databases to target Pdia4 and is also differentially expressed after cyclosporin A treatment. Pdia4 is involved in protein folding and secretion in the endoplasmic reticulum, confirming the results in the previous chapters that endoplasmic reticulum stress is involved in drug-induced hepatotoxicity. A network is created using the differentially expressed proteins and interacting

genes and microRNAs are added. The network consists of 6 clusters, which are involved in toxicological mechanisms, including protein folding and secretion. Differentially expressed metabolites indicate changes in glucose, lipid and cholesterol homeostasis. The findings in this chapter suggest that microRNAs may be involved in the mechanisms underlying drug-induced hepatotoxicity.

MicroRNAs specific for the liver are proposed as early biomarkers of liver injury, e.g. miR-122. [10] However, the role of microRNAs in drug-induced hepatotoxicity is largely unknown. In **Chapter 6** we expose HepG2 cells and primary mouse hepatocytes to three well known human hepatotoxicants, cyclosporin A, amiodarone and acetaminophen, which are known to induce cholestasis, steatosis and necrosis, respectively. MicroRNA and mRNA expression is analyzed using microarrays, and reveals multiple differentially expressed microRNAs and mRNAs overlapping between all treatments. Investigation of the overlapping microRNAs and their target genes indicates that regeneration and proliferation are involved in the cellular reaction to hepatotoxic compounds. A possible role could be the induction of cell repair and inhibition of apoptosis. However, since these three compounds induce different subtypes of liver injury, most significant changes of microRNAs and mRNAs are drug-specific. Investigation of drug-specific changes reveals a possible function of microRNAs in the metabolization of these drugs. Furthermore, differentially expressed microRNAs may be involved in lipid homeostasis and cholestatic mechanisms. Targeted research of the drug-specific differentially expressed microRNAs, their target genes and the proteins they code for may further unravel the role of microRNAs in drug-induced hepatotoxicity.

Limitations and future recommendations

Promising results are achieved for the classification of hepatotoxicants using HepG2 cells, but it should be taken into account that only a limited amount of compounds are tested. Validation efforts should thus be performed using more hepatotoxic and non-hepatotoxic compounds. Furthermore, HepG2 cells show low expression of multiple important genes, which complicates an in depth analysis of mechanisms of liver toxicity. The metabolic competence of primary mouse hepatocytes is comparable to primary human hepatocytes, which makes these more suitable for the investigation of mechanisms underlying drug-induced hepatotoxicity. However, rodent hepatocytes are not human hepatocytes, and extrapolating the results found in rodents, to the human situation has been shown to be challenging. [11] Furthermore, hepatocyte cultures only consist of one cell type, where in the liver multiple cell types are present, e.g. Kupffer cells and hepatic stellate cells which play important roles in inflammation and fibrosis. Since these processes are important in hepatotoxicity [12], future experiments should preferably include these cells.

Three dimensional, organotypical cell cultures have recently been developed and seem to better resemble the *in vivo* liver. [13] Furthermore, pluripotent stem cells can be used to produce metabolic competent human hepatocytes. [14] Further improvement of these techniques could result in better liver models, which will result in more reliable human toxicity screening. [15]
Our results show that multi-omics analysis is capable of providing more relevant information regarding the induced hepatotoxicity by compounds. However, the integrated analysis of multiple omics is difficult since the available tools are not able to combine all omics data. The development of new tools that are capable of integrating all omics platforms has the potential to further deepen our knowledge on drug-induced liver injury.

The difference in numbers of investigated genes, proteins and metabolites further challenges the integration. Microarray analysis, and even more next generation sequencing, can measure the expression of thousands genes at once, which provides valuable data on the transcriptional changes induced by hepatotoxicants. However, the field of proteomics and metabonomics fall behind. The complete human proteome and metabolome are not published yet; however, recent publications show great progress. [16-17] Future research should result in high-throughput platforms for complete proteome and metabolome analyses, which would help to further unravel the mechanisms underlying drug-induced toxicity.

Conclusion

The results described in this thesis show that HepG2 cells can be used to classify hepatotoxic and non-hepatotoxic compounds and provide information on the mechanisms underlying drug-induced hepatotoxicity. The drug-induced changes in HepG2 cells and primary mouse hepatocytes indicate that endoplasmic reticulum stress and the unfolded protein response are important events in hepatotoxicity. The research in this thesis describes an integrated analysis of multiple omics techniques, which results in more relevant information on drug-induced toxicity compared to the single omics analyses. Furthermore, microRNAs have been demonstrated to play an important role in drug-induced toxicity.

This thesis thus lays the foundation for the further development of integrated multi-omics analyses in *in vitro* toxicity screenings, which will improve the classification of hepatotoxic compounds and the knowledge on drug-induced hepatotoxicity.

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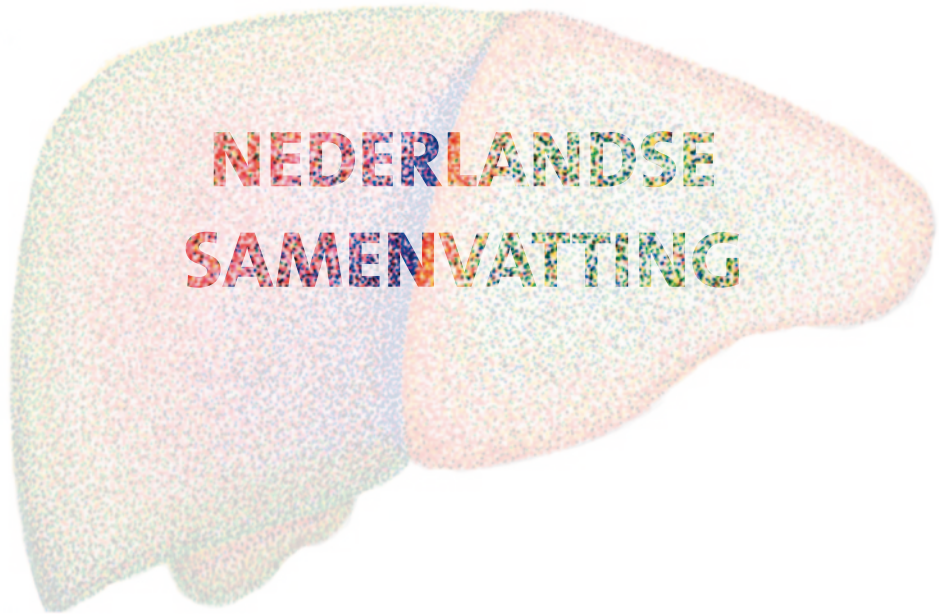

Van den Hof, W.F.P.M.

Het onderzoek dat wordt beschreven in dit proefschrift, werd uitgevoerd om te onderzoeken of *in vitro* levermodellen gebruikt kunnen worden voor het classificeren van schadelijke stoffen voor de lever en om de fundamentele mechanismen te ontrafelen van leverschade veroorzaakt door deze schadelijke stoffen. De huidige screening van nieuwe medicijnen is afhankelijk van dierproeven; echter, het feit dat een stof als veilig wordt beoordeeld op basis van dergelijke dierproeven, voorkomt niet dat sommige kandidaat-geneesmiddelen in klinische studies of zelfs na introductie op de markt toch giftig blijken te zijn voor de mens.[1] De lever is het belangrijkste orgaan met betrekking tot het metabolisme en de uitscheiding van medicijnen, waardoor dit orgaan extra kwetsbaar is voor schade geïnduceerd door medicijnen. Er is behoefte aan betrouwbare *in vitro* testen voor toxiciteit met een hoge doorvoersnelheid, voornamelijk omdat het aantal kandidaat-geneesmiddelen elk jaar stijgt. Er kan onderscheid gemaakt worden tussen verschillende klassen van leverschade, waaronder necrose, steatose en cholestase, en elk van deze klassen heeft een ander fenotypische profiel. Necrose is een vorm van vroegtijdige celdood, steatose wordt gekenmerkt door de ophoping van vetten in de cel en stapeling van gal bestanddelen, zoals galzouten, is kenmerkend voor cholestase.[2-4] Met behulp van traditionele biomarkers voor leverbeschadiging is de differentiatie tussen verschillende klassen van leverschade moeilijk. Daarom wordt er veel energie gestoken in het ontwikkelen van nieuwe testmethodes om nieuwe en meer accurate biomarkers te vinden. Binnen het gebied van toxicogenomics zijn er verschillende platforms voor het genereren van moleculaire informatie. Het genoom-breed profileren van RNA-moleculen, zoals messenger RNA (mRNA) en microRNA, is beter bekend als transcriptomics en vertegenwoordigt het meest gebruikte platform in toxicogenomics onderzoek. Proteomics wordt gebruikt om de vertaling van mRNA in eiwitten en post-translationele modificaties te onderzoeken. Metabonomics onderzoekt het niveau van metabolieten. Al deze platformen leveren individueel waardevolle informatie op over cellulaire reacties na blootstelling aan giftige stoffen; echter, een gecombineerde analyse van alle platformen heeft de potentie om de fundamentele mechanismen van leverschade veroorzaakt door medicijnen volledig op te helderen. Om leverschade veroorzaakt door medicijnen *in vitro* te onderzoeken, worden meerdere modellen voorgesteld als relevant alternatief voor de *in vivo* situatie in laboratoriumdieren. Leverplakken houden een driedimensionale configuratie en bevatten alle celtypes en functies van de intacte lever.[5] Echter, het voorbereiden en kweken van deze leverplakken is bewerkelijk en langdurige blootstellingen zijn onmogelijk. Primaire humane hepatocyten worden beschouwd als een van de beste *in vitro* levermodellen, omdat deze cellen grotendeels hetzelfde gedrag vertonen als hepatocyten *in vivo*.[6] Echter humane leverdonoren zijn schaars en door interindividuele variatie is de vergelijking van meerdere experimenten moeilijk. Stamcellen kunnen gebruikt worden om een onbeperkte hoeveelheid aan primaire hepatocyten te verkrijgen en zijn daarom mogelijk een waardevol alternatief model. Tot nu toe belemmeren moeilijkheden met kweken en differentiatie protocollen het gebruik van hepatocyten verkregen van stamcellen in toxiciteit screenings.

Onderzoek naar lever toxiciteit met behulp van transcriptomics

Andere lever modellen die het onderzoeken waard zijn, zijn geïmmortaliseerde cellijnen waarvan HepG2- en HepaRG-cellen het meest gebruikt worden. Beide cellijnen zijn menselijke levercellen en zijn eenvoudig en goedkoop te kweken. HepG2 cellen vertonen lagere referentiewaarden van fase I en fase II-enzymen dan HepaRG, maar de reactie op levertoxische stoffen is vergelijkbaar. [7] **In Hoofdstuk 2** trachten we daarom het principe te bewijzen dat HepG2 cellen gebruikt kunnen worden om bekende levertoxische en niet-levertoxische stoffen te classificeren op hun onderscheidende genexpressie profielen. Voor de classificatie gebruiken we 'Prediction Analysis for Microarrays'. Op basis van 36 genen, zijn we in staat om levertoxische en niet-levertoxische stoffen te classificeren met een nauwkeurigheid van 92% voor de training set en 91% voor de validatie set. In een poging cholestatische verbindingen te subclassificeren, is een tweede classificatiemodel opgezet. Deze subclassificatie van cholestatische stoffen resulteert in een nauwkeurige voorspelling van 8 van de 9 cholestatische verbindingen met een totale nauwkeurigheid van 93%, op basis van 12 genen. De genen die zijn geselecteerd voor de classificatie van hepatotoxiciteit en cholestase, wijzen op de mogelijke betrokkenheid van endoplasmatisch reticulum stress en een ongevouwen eiwit respons in leverschade veroorzaakt door medicijnen. Hoewel we in staat zijn om nauwkeurig levertoxische stoffen te classificeren op basis van transcriptoom analyse van HepG2-cellen, moet het aantal verbindingen verhoogd worden voor het verbeteren en valideren van deze classificatie methode.

Cholestase veroorzaakt door medicijnen heeft een hoog sterftecijfer en is een van de meest ernstige manifestaties van leverschade veroorzaakt door medicijnen. Remming van transporteiwitten waardoor intracellulaire accumulatie van gal optreedt, is voorgesteld als het onderliggende mechanisme. Wij streven dan ook naar de evaluatie van drie *in vitro* levermodellen voor het onderzoek naar cholestase veroorzaakt door medicijnen door deze te vergelijken met cholestatische patiënten, zoals beschreven staat in **Hoofdstuk 3**. Microarray data van HepG2 cellen, primaire muis hepatocyten en primaire humane hepatocyten blootgesteld aan drie cholestatische verbindingen, cyclosporine A, chloorpromazine en ethinylestradiol, zijn vergeleken met transcriptoom data van cholestatische patiënten. Om een *in vitro* vingerafdruk van cholestase veroorzaakt door medicijnen te vinden zijn de differentieel tot expressie komende genen onderzocht die overlappen in alle behandelingen. Slechts een beperkt aantal significant veranderde genen lijken te overlappen in alle drie de behandelingen in primaire muis en humane hepatocyten, en geen van deze komen differentieel tot expressie in de *in vivo* monsters. Behandeling van HepG2 cellen met deze drie verbindingen resulteert echter in 151 differentieel tot expressie komende genen die overlappen in alle drie de behandelingen. 13 van deze genen komen ook differentieel tot expressie in de *in vivo* cholestase monsters en deze genen zijn betrokken bij een ongevouwen eiwit respons. Dit is in overeenstemming met de resultaten van **Hoofdstuk 2**, die suggereren dat endoplasmatisch reticulum stress en een ongevouwen eiwit respons een belangrijke rol spelen bij hepatotoxiciteit, vooral in cholestase. De beperkte overlap van differentieel tot expressie komende genen na behandeling met deze drie cholestatische verbindingen

wijst erop dat er verschillende mechanismen betrokken zijn bij de geïnduceerde toxiciteit. Een eerder gepubliceerde metabole route voor cholestase veroorzaakt door medicijnen, is vervolgens gebruikt om de genexpressie-veranderingen in humane *in vivo* cholestase te visualiseren. Genen die betrokken zijn bij de opname en de synthese van galzouten worden geremd en de transcriptie van hepatocellulaire export eiwitten word gestimuleerd, wat wijst op een adaptieve respons van de zieke lever om het niveau van intracellulaire galzouten te verlagen en verdere stapeling van galzouten te voorkomen. Indicaties voor een adaptieve respons worden ook waargenomen in de *in vitro* modellen na behandeling met de cholestatische stoffen. Bovendien worden medicijn-specifieke veranderingen in de expressie van genen in de metabole route voor cholestase waargenomen die kunnen leiden tot intracellulaire accumulatie van galzouten.

Multi-omics analyse in hepatotoxiciteit

Met het oog op het ontrafelen van de fundamentele mechanismen van toxiciteit veroorzaakt door medicijnen, is een multi-omics aanpak uitgevoerd in **Hoofdstuk 4**. HepG2 cellen zijn behandeld met de cholestatische verbinding cyclosporine A, waarna het mRNA, microRNA, en metabonoom profiel is onderzocht. Behandeling van HepG2-cellen zorgt voor een afname van de intracellulaire galzoutconcentratie en induceert een intracellulaire accumulatie van cholesterol. Analyse van metabole routes is uitgevoerd met behulp van de differentieel tot expressie komende genen en significant veranderde metabolieten. Een geïntegreerde analyse resulteert in meer relevante informatie dan de afzonderlijke analyses van de twee datasets. Geïntegreerde analyse van metabole routes wijst op een significante verandering van routes betrokken bij celcyclus en cellulaire stofwisseling. Bovendien zijn significante veranderingen gevonden die betrokken zijn bij de eiwit verwerking van het endoplasmatisch reticulum, de biosynthese van galzuren en het cholesterol metabolisme. Verder is de route voor cholestase veroorzaakt door medicijnen, beschreven in **Hoofdstuk 3**, aangevuld met mogelijke interacties tussen genen en microRNAs. Analyse van de genen, microRNAs en metabolieten van deze route levert waardevolle informatie op over de cholestatische eigenschappen van verbindingen. Na validatie kan deze route gebruikt worden om kandidaat-geneesmiddelen te screenen op hun cholestase-inducerende potentie. De bevindingen in dit hoofdstuk bevestigen dat een geïntegreerde omics benadering, waarbij transcriptoom en metabonoom analyses van HepG2 cellen worden gecombineerd, verdere ontrafeling van de fundamentele mechanismen van hepatotoxiciteit veroorzaakt door cyclosporine A mogelijk maakt.

Er zijn echter meerdere belangrijke genen die minimaal tot expressie komen in HepG2 cellen, waaronder genen betrokken bij galzuursynthese en belangrijke export eiwitten waaronder de galzout export pomp. Tot nu toe worden toxiciteit screenings meestal uitgevoerd op knaagdieren, wat suggereert dat de primaire hepatocyten van knaagdieren een relevant *in vitro* lever model vormen. Eerder onderzoek geeft aan dat de primaire muis hepatocyten metabolisch stabieler zijn dan primaire rat hepatocyten. [8] Primaire muis hepatocyten zijn daarom gebruikt in het onderzoek beschreven in **Hoofdstuk 5**, om

de fundamentele mechanismen van toxiciteit veroorzaakt door cyclosporine A verder te ontrafelen. Na behandeling met cyclosporine A, is de expressie van eiwitten, mRNA, microRNA en metabolieten geanalyseerd. Geïntegreerde analyse van significant veranderde eiwitten en genen laat zien dat de expressie van eiwit-disulfide isomerase familie A, lid 4 (Pdia4) aanzienlijk gestimuleerd wordt op zowel eiwit-, als mRNA-niveau. MicroRNAs kunnen de stabiliteit en de vertaling van mRNA beïnvloeden en er bestaan verschillende databases met experimenteel gevalideerde en voorspelde interacties van microRNAs met mRNAs. [9] In deze studie, zijn geen experimenteel gevalideerde interacties geïdentificeerd van microRNAs met het mRNA van Pdia4. Een interactie tussen mmu-miR-182-5p en het mRNA van Pdia4 wordt voorspeld door twee afzonderlijke databases en dit microRNA komt ook differentieel tot expressie na behandeling met cyclosporine A. Pdia4 is betrokken bij het vouwen en de secretie van eiwitten in het endoplasmatisch reticulum, wat bevestigd dat endoplasmatisch reticulum stress mogelijk betrokken is bij hepatotoxiciteit veroorzaakt door medicijnen. Een netwerk is gemaakt met behulp van de differentieel tot expressie komende eiwitten en de interacties met genen en microRNAs zijn toegevoegd. Het netwerk bestaat uit 6 clusters, die betrokken zijn bij verschillende toxicologische mechanismen, waaronder het vouwen en de secretie van eiwitten. Differentieel tot expressie komende metabolieten weerspiegelen veranderingen in de homeostase van glucose, lipiden en cholesterol. De bevindingen in dit hoofdstuk suggereren dat microRNAs een rol kunnen spelen in de fundamentele mechanismen van hepatotoxiciteit veroorzaakt door medicijnen.

Onderzoek suggereert dat microRNAs specifiek voor de lever, zoals miR-122, gebruikt kunnen worden als vroege biomarkers voor leverschade. [10] Echter, de rol van microRNAs in hepatotoxiciteit veroorzaakt door medicijnen is grotendeels onbekend. In **Hoofdstuk 6** worden HepG2 cellen en primaire muis hepatocyten blootgesteld aan drie stoffen die toxisch zijn voor de humane lever, cyclosporine A, amiodarone en paracetamol, waarvan bekend is dat deze respectievelijk cholestase, steatose en necrose veroorzaken. MicroRNA en mRNA expressie is geanalyseerd met behulp van microarrays en onthult dat meerdere differentieel tot expressie komende microRNAs en mRNAs overlappen tussen alle behandelingen. Onderzoek van de overlappende microRNAs en de genen waarmee ze een interactie vertonen, toont aan dat regeneratie en proliferatie betrokken zijn bij de cellulaire reactie op hepatotoxische stoffen. Een mogelijke rol kan de inductie van cel reparatie en remming van apoptose zijn. Echter, aangezien deze drie verbindingen verschillende subtypes van leverbeschadiging veroorzaken, zijn de belangrijkste geïnduceerde veranderingen van microRNAs en mRNAs medicijn-specifiek. Onderzoek naar medicijn-specifieke veranderingen onthult een mogelijke functie van microRNAs in het metabolisme van deze medicijnen. Bovendien zijn differentieel tot expressie komende microRNAs wellicht betrokken bij de homeostase van vetten en bij cholestatische mechanismen. Gericht onderzoek naar de medicijn-specifieke microRNAs die differentieel tot expressie komen, de genen waarmee ze een interactie vertonen en de eiwitten waar deze genen voor coderen, zouden de rol van microRNAs in hepatotoxiciteit veroorzaakt door medicijnen verder kunnen ontrafelen.

Beperkingen en aanbevelingen voor de toekomst

Veelbelovende resultaten zijn bereikt voor de classificatie van lever-toxische stoffen met behulp van HepG2 cellen, maar er moet rekening gehouden worden met het feit dat slechts een beperkte hoeveelheid verbindingen getest zijn. Verdere validatie moet dus worden uitgevoerd met meer hepatotoxische en niet-hepatotoxische verbindingen. Bovendien vertonen HepG2 cellen een lage expressie van meerdere belangrijke genen, die een diepgaande analyse van de onderliggende mechanismen van lever-toxiciteit compliceert. De metabolische competentie van primaire muis hepatocyten is vergelijkbaar met primaire humane hepatocyten, waardoor deze beter geschikt zijn voor het onderzoek naar de onderliggende mechanismen in hepatotoxiciteit veroorzaakt door medicijnen. Echter, hepatocyten van knaagdieren zijn geen humane hepatocyten en extrapolatie van de resultaten gevonden bij knaagdieren naar de humane situatie blijkt een hele uitdaging. [11] Verder bestaan *in vitro* kweken van hepatocyten slechts uit één celtype, waar in de lever verschillende celtypen aanwezig zijn, waaronder kupffercellen en stellaatcellen die een belangrijke rol spelen bij ontstekingen en fibrose. Aangezien deze processen een belangrijke rol spelen bij hepatotoxiciteit, dienen toekomstige *in vitro* experimenten bij voorkeur ook deze cellen te bevatten. [12]

Drie dimensionale, organotypische celculturen zijn recentelijk ontwikkeld en lijken beter op de *in vivo* lever. [13] Verder zouden pluripotente stamcellen gebruikt kunnen worden om metabolisch competente menselijke hepatocyten te produceren. [14] Verdere verbetering van deze technieken kan leiden tot betere levermodellen wat zal resulteren in een hogere betrouwbaarheid van humane toxiciteit screening. [15]

Onze resultaten tonen aan dat een geïntegreerde analyse van meerdere omics meer relevante informatie oplevert over de hepatotoxiciteit veroorzaakt door medicijnen. Echter, een geïntegreerde analyse van meerdere omics is moeilijk aangezien de beschikbare software nog niet in staat is om alle omics data te combineren. De ontwikkeling van nieuwe software die in staat is om data van alle omics platforms te integreren, heeft het potentieel om onze kennis van leverschade veroorzaakt door medicijnen verder te verdiepen.

Het verschil in aantallen tussen de onderzochte genen, eiwitten en metabolieten is een extra uitdaging bij de integratie. Microarray analyse, en in het bijzonder next generation sequencing, kan de expressie van duizenden genen tegelijkertijd meten, en levert waardevolle informatie op over transcriptionele veranderingen bij leverschade. Echter, proteomics en metabonomics lopen hierbij wat achter. Het volledige menselijk proteoom en metaboloom zijn nog niet gepubliceerd, maar recente publicaties tonen grote vooruitgang. [16-17] Toekomstig onderzoek moet resulteren in een platform met een hoge doorvoercapaciteit voor complete proteoom en metaboloom analyses wat zou helpen bij het ontrafelen van de fundamentele mechanismen van hepatotoxiciteit veroorzaakt door medicijnen.

Conclusie

De in dit proefschrift beschreven resultaten laten zien dat HepG2 cellen gebruikt kunnen worden om hepatotoxische en niet-hepatotoxische stoffen te classificeren en informatie te verstrekken over de fundamentele mechanismen van hepatotoxiciteit veroorzaakt door medicijnen. De veranderingen in HepG2 cellen en primaire muis hepatocyten veroorzaakt door toxische medicijnen, duiden erop dat endoplasmatisch reticulum stress en een ongevouwen eiwit respons belangrijke gebeurtenissen zijn in hepatotoxiciteit. Het onderzoek in dit proefschrift beschrijft een geïntegreerde analyse van meerdere omics technieken, die resulteert in meer relevante informatie over toxiciteit veroorzaakt door medicijnen dan enkelvoudige omics analyses. Bovendien is aangetoond dat microRNAs mogelijk een belangrijke rol spelen bij toxiciteit veroorzaakt door medicijnen.

Dit proefschrift legt daarmee de basis voor de verdere ontwikkeling van geïntegreerde multi-omics analyses in *in vitro* toxiciteit screenings, die de classificatie van hepatotoxische stoffen en de kennis van hepatotoxiciteit veroorzaakt door medicijnen zal verbeteren.

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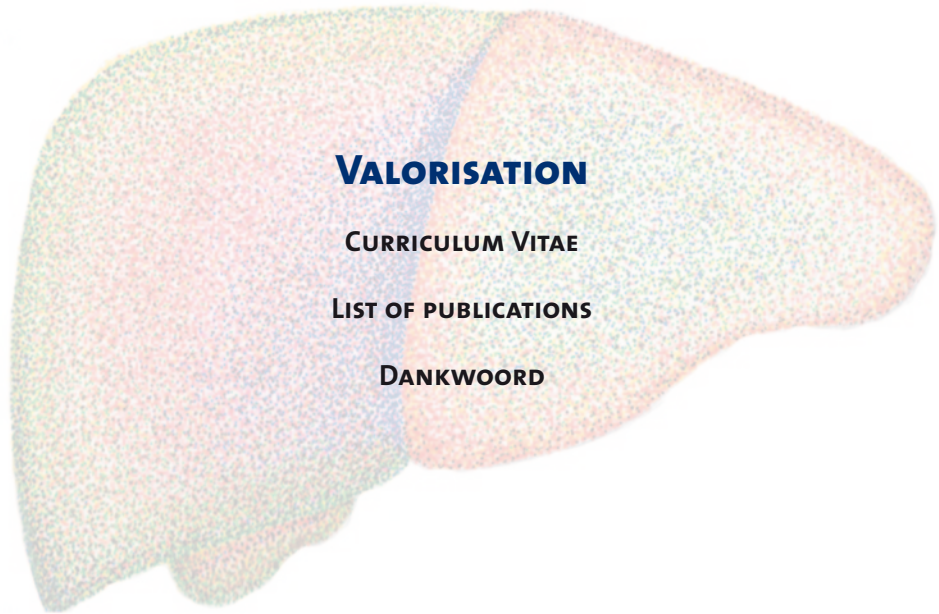

Valorisation

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Valorisation

The creation of value out of knowledge, valorisation, has two dimensions: an economic aspect and a societal aspect. The economic aspect of value-creation of our research is highlighted first. The development of a new drug may easily take several years and it may cost up to US $ 800 million before approval and release onto the market, making it a time- and money-consuming business. [1] Before new drugs progress into clinical trials, preclinical testing is performed, which mostly consists of long-term animal experiments. If a drug does not induce adverse effects in these animal tests, the drug will be tested in clinical trials. Approximately 20% of all new drugs which enter clinical trials, are proven successful and advance onto the market. [2] However, a large number of drug candidates fail during preclinical tests or clinical trials. The most indicated reason for termination of these projects is the lack of efficacy of the new drug candidate. The second most indicated reason is drug-induced toxicity. [3] Liver injury is reported in approximately 30% of all cases of drug-induced toxicity in clinical trials, which indicates that drug-induced hepatotoxicity is one of the major reasons for drug withdrawal. [4-6] Since the liver is the most important organ involved in the metabolization and secretion of drugs, accumulation of drugs or toxic drug metabolites may induce adverse drug effects. Despite the fact that drug candidates are extensively tested in preclinical screenings and clinical trials, some drugs are found to be hepatotoxic after admittance onto the market. This indicates that preclinical testing needs to be improved to reduce the number of hepatotoxic drug candidates advancing into animal experiments and clinical trials.

Previous publications report an accurate classification of non-hepatotoxicants and hepatotoxic compounds, including discrimination of different classes of toxicants, based on the *in vivo* gene expression profiles in rat livers. [7, 8] Prediction of genotoxic properties of compounds *in vivo* could successfully be predicted *in vitro* using gene expression analysis combined with the Ames mutagenicity test. [9] In chapter 2 of this thesis we describe a transcriptomic investigation of drug-induced toxicity in an *in vitro* liver cell line, which resulted in a selection of genes capable of classifying *in vivo* liver toxicants and non-toxicants. An accuracy of 91% was obtained in the classification of hepatotoxicants and non-hepatotoxicants, using the expression of 36 genes. Furthermore, using 12 selected genes in a second model, we were able to classify cholestatic properties of compounds with an accuracy of 93%. These findings may result in the development of a sensitive *in vitro* test to screen for drug-induced toxicity, which in time may result in a reduction and replacement of laboratory animals in toxicity screening. Drug development industry could save a lot of money and time if new drug candidates could be screened in a fast and accurate *in vitro* test instead of in long-term toxicity testing in laboratory animals. *In vitro* testing allows testing of drug candidates in a high throughput way, which prevents spending too much time and money on toxic candidates in an early stage of development.

The selected genes could be used to develop an assay to detect hepatotoxicity which can

be commercialized. The assay could consist of a microarray-based setup containing probes for the measurement of the selected classifiers. Users would expose an *in vitro* liver cell line to the drug candidates which they want to test, and isolate the RNA. The isolated RNA is applied to the probes and measurement of fluorescent intensity is used to quantify the expression of the selected genes relative to a negative control. For this, also automated data analysis pipelines should be put in place. Hepatotoxic properties will be reported based on the up or down-regulation of the genes as a reaction to the exposure of the drug-candidates. In order to valorize this assay, the used protocol should be patented.

However, the classification assay described in Chapter 2 was set-up and validated using a limited amount of compounds. Therefore, the selected genes should be validated using a large number of hepatotoxic and non-hepatotoxic compounds before these can be used in a new hepatotoxicity test. Personnel, money and time are needed to expose cells to all these compounds. Approximately 2-3 persons are required for a 1-2 year project for the coordination of compound recruitment, the exposure of cells, microarray analyses, data processing and the statistical analyses. Applying for dedicated grants (e.g. STW Valorisation Grant) to get money for the development of this assay would be one of the possibilities. Furthermore, collaborating with the drug development industry could be an option. Therefore, implementation of this new assay is fully dependent on the time it would take to raise enough money for the measurement of more compounds in order to validate the genes selected for classification.

Furthermore, the research described in Chapters 4, 5 and 6 emphasize the added value of integrating multiple omics in order to fully elucidate the mechanistic understanding of toxicity profiles. In these chapters, data from multiple omics techniques are combined in two *in vitro* liver models, HepG2 cells and primary mouse hepatocytes. In Chapter 4, HepG2 cells were treated with cyclosporin A and we show that integrating transcriptomics and metabonomics results in more information on the mechanisms underlying cyclosporin A-induced toxicity. This was confirmed in the research described in Chapter 5, using primary mouse hepatocytes, in which transcriptomics was integrated with proteomics and metabonomics. In Chapter 6, mRNA and microRNA profiling is integrated which provides more information on the possible influence of microRNAs on the translation of drug-induced effects on mRNA level.

The transcriptomics and metabonomics data described in Chapter 4 was used for integrated pathway analysis using the available open source tool IMPaLA. [10] MicroRNA data was integrated with transcriptomics data in Chapters 4, 5 and 6 using experimentally validated target genes from miRTarBase and the overlap of predicted target genes from miRanda and TargetScan. [11-13] For the visualization of different omics data, PathVisio was used. [14] These open source tools and databases provide valuable information on the interactions between the different omics data. However, no tools are available yet to integrate all omics data. In order to valorize the research described in these chapters, the results of the integrated analyses should be used to further improve existing tools or to set-up a new open source tool for multi-omics analysis.

Approximately 2 persons with statistical and programming skills are required for a 1-2 year project for the coordination of data recruitment and processing, statistical analysis and the set-up of the scripts needed to run such a tool. Once the tool is operational, time and money could be invested in the web-design of the open source tool. Collaboration with the research groups that developed already existing open source tools, like IMPaLA and PathVisio, could be an option. Especially the expansion of functions in PathVisio would be interesting, since separate plugins can be developed and integrated into the tool. In order to finance this project, grants applications are needed.

Apart from the economic aspect, societal value can be created from knowledge. Chemical safety is important and increasing the safety of chemicals is a task of regulatory authorities like the Organisation for Economic Co-operation and Development (OECD). The OECD launched a new program in 2012 on the generation of Adverse Outcome Pathways (AOPs). The integration of different omics data will facilitate the construction of AOPs. AOPs support the integration of different kinds of data and provide a mechanistic basis for drug-induced toxicity. Therefore, AOPs can be used to support the assessment of the toxic potential of compounds. [15] The results of the research described in this thesis should be used to develop new AOPs and expand existing ones. Vinken et al. published an AOP for cholestasis induced by the inhibition of the Bile Salt Export Pump (BSEP) by drugs. [16] In the research described in Chapter 4 and 5, HepG2 cells and primary mouse hepatocytes are treated with the BSEP-inhibiting drug cyclosporin A. The results of these multi-omics approaches indicate the possible involvement of endoplasmic reticulum stress and the unfolded protein response in cholestasis. Ones validated, these mechanisms should be added to the AOP for cholestasis.

The datasets presented in this thesis, especially the data from experiments with multiple time points and doses, should be further explored in order to construct AOPs for the different adverse outcomes in drug-induced hepatotoxicity. The data presented in this thesis should be supplemented with additional datasets, including different kinds of data like e.g. physiologically based biokinetic modeling. Focusing on 1 adverse outcome will increase the feasibility of such a project. Approximately 2 persons will be required for a 1-2 year project in order to collect available data and construct the AOP. Follow-up projects for other adverse outcomes will use the developed pipeline of the first project. The developed AOPs can be used to identify key events in the induction of adverse outcomes, which will enable the use of *in vitro* and/or in silico tests to assess the ability of drugs to induce the adverse outcomes. These tests will reduce the amount of laboratory animals needed for toxicity testing and decrease the number of toxic drugs advancing into clinical trials and onto the market, which highlights the societal aspect of our research.

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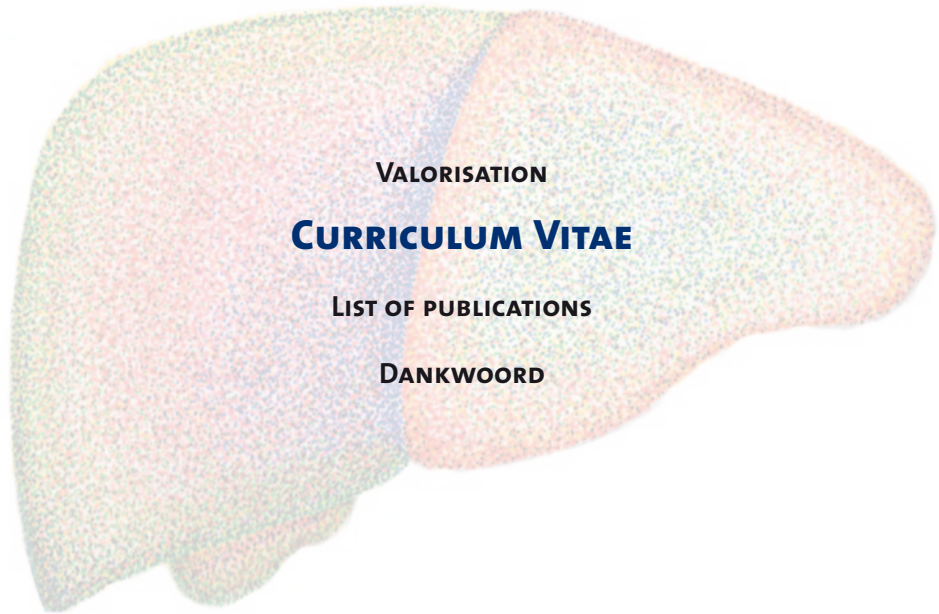

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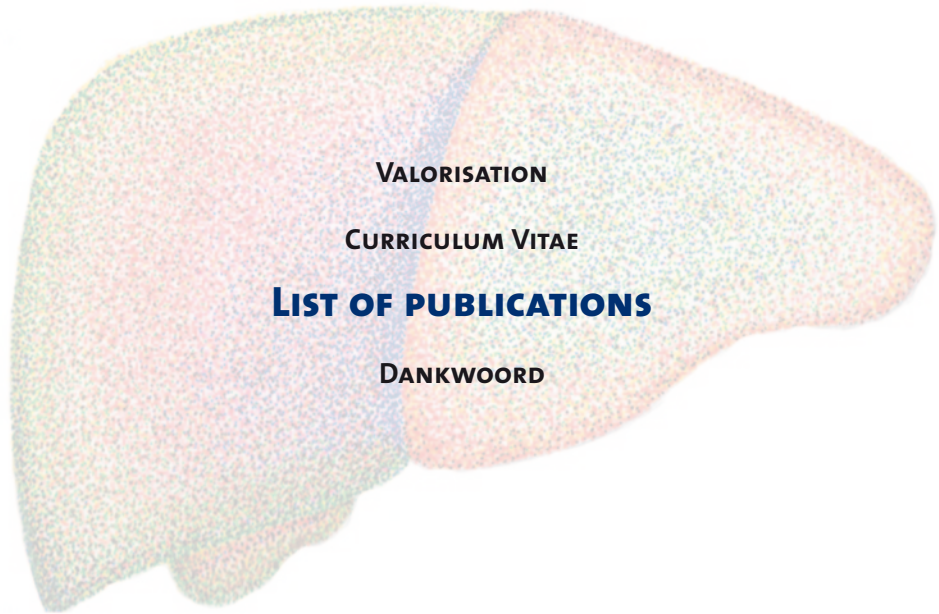
Curriculum Vitae

Wilhelmus Franciscus Petrus Maria Van den Hof was born on September 21st 1984 in Heerlen, the Netherlands. After finishing secondary school in the disciplines “Nature and Health” and “Nature and Technology” at the Jeanne d’Arc College in Maastricht, he started the bachelor study “Biology and Medical Laboratory Research” in 2001 at Zuyd University of Applied Sciences in Heerlen. He did his internship at Atrium Medical Center in Heerlen where he investigated the analytical interference of lipemia on the analysis of patient samples. He obtained his Bachelor of Applied Sciences degree in 2006, after which he worked for 1 year as a laboratory technician at the Clinical Chemistry department at the Maastricht University Medical Center.

He continued his education in 2007, when he started with a Master’s program in Biomedical Sciences with a specialization in Clinical Molecular Sciences at Maastricht University. He did his internship at the Clinical Chemistry department at the Maastricht University Medical Center, where he investigated the degradation of cardiac troponin and the influence on test harmonization. During his master he obtained licenses for working with radioactive materials (Radiation hygiene, expert level 5b) and laboratory animals (according to art. 9 of the Dutch law on experimental animals).

In October 2009 he started as a PhD candidate at the department of Health Risk Analysis and Toxicology at Maastricht University, now the department of Toxicogenomics. Under supervision of Prof. Dr. J. Kleinjans, Dr. W. Wodzig and Dr. J. van Delft he performed the research presented in this thesis entitled: “*In vitro* toxicogenomics, unravelling the mechanisms underlying drug-induced hepatotoxicity”. From May 2014 onwards he has been working on the project “Systems toxicology supported data infrastructure for human risk assessment” at the department of Toxicogenomics at Maastricht University. This project will continue the development of the ‘ASAT Knowledge Base’, which is based upon the ‘Assuring Safety without Animal Testing’ (ASAT) principle, in which human disease data is integrated with data from non-animal *in vitro* models.



Valorisation

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Full Papers

Van den Hof, W.F.P.M., Coonen, M.L.J., van Herwijnen, M., Brauers, K., Wodzig, W.K.W.H., van Delft, J.H.M., Kleinjans, J.C.S. (2014) Classification of Hepatotoxicants Using HepG2 Cells: A Proof of Principle Study. Chem. Res. Toxicol., 27, 433-442.

Van den Hof, W.F.P.M., Van Summeren, A., Lommen, A., Coonen, M.L.J., Brauers, K., van Herwijnen, M., Wodzig, W.K.W.H., Kleinjans, J.C.S. (2014) Integrative cross-omics analysis in primary mouse hepatocytes unravels mechanisms of cyclosporin A-induced hepatotoxicity. Toxicology, 324, 18-26.

Van den Hof, W.F.P.M., Ruiz-Aracama, A., Van Summeren, A., Jennen, D.G.J., Gaj, S., Coonen, M.L.J., Brauers, K., Wodzig, W.K.W.H., van Delft, J.H.M., Kleinjans, J.C.S. Integrating multiple omics to unravel mechanisms of Cyclosporin A induced hepatotoxicity *in vitro*. *Under review.*

Van den Hof, W.F.P.M., Coonen, M.L.J., van Herwijnen, M., Brauers, K., Wodzig, W.K.W.H., Olde Damink, S.W.M., Schaap, F.G., Kleinjans, J.C.S. Validation of gene expression profiles from cholestatic hepatotoxicants *in vitro* against human *in vivo* cholestasis. *In preparation.*

Van den Hof, W.F.P.M., Coonen, M.L.J., Van Summeren, A., van Herwijnen, M, Brauers, K., Wodzig, W.K.W.H., Kleinjans, J.C.S. MicroRNA Involvement in Drug-Induced Hepatotoxicity: A multi-omics approach. *In preparation.*

Abstracts

Van den Hof, W.F.P.M., Coonen, M.L.J., van Herwijnen, M., Brauers, K., Wodzig, W.K.W.H., van Delft, J.H.M., Kleinjans, J.C.S. Classification of Hepatotoxicants Using HepG2 Cells: A Proof of Principle. Poster presentation at the GROW Science Day, November 2013, Maastricht, the Netherlands.

Van den Hof, W.F.P.M., Ruiz-Aracama, A., Van Summeren, A., Jennen, D.G.J., Gaj, S., Coonen, M.L.J., Brauers, K., Wodzig, W.K.W.H., van Delft, J.H.M., Kleinjans, J.C.S. A systems biology approach to unravel mechanisms of Cyclosporine A induced hepatotoxicity. Poster presentation at the Systems Toxicology Conference, April 2013, Ascona, Switzerland.

Van den Hof, W.F.P.M., Ruiz-Aracama, A., Van Summeren, A., Jennen, D.G.J., Gaj, S., Coonen, M.L.J., Brauers, K., Wodzig, W.K.W.H., van Delft, J.H.M., Kleinjans, J.C.S. Integrating multiple omics to unravel mechanisms of cyclosporine A induced hepatotoxicity. Oral presentation at the Annual meeting of the Netherlands Toxicogenomics Centre (NTC), January 2013, Amsterdam, The Netherlands.

Van den Hof, W.F.P.M., Ruiz-Aracama, A., Van Summeren, A., Jennen, D.G.J., Gaj, S., Coonen, M.L.J., Brauers, K., Wodzig, W.K.W.H., van Delft, J.H.M., Kleinjans, J.C.S. A systems biology approach to unravel mechanisms of Cyclosporine A induced hepatotoxicity. Poster presentation at the First Joint German-Dutch Meeting of the Societies of Toxicology, October 2012, Düsseldorf, Germany.

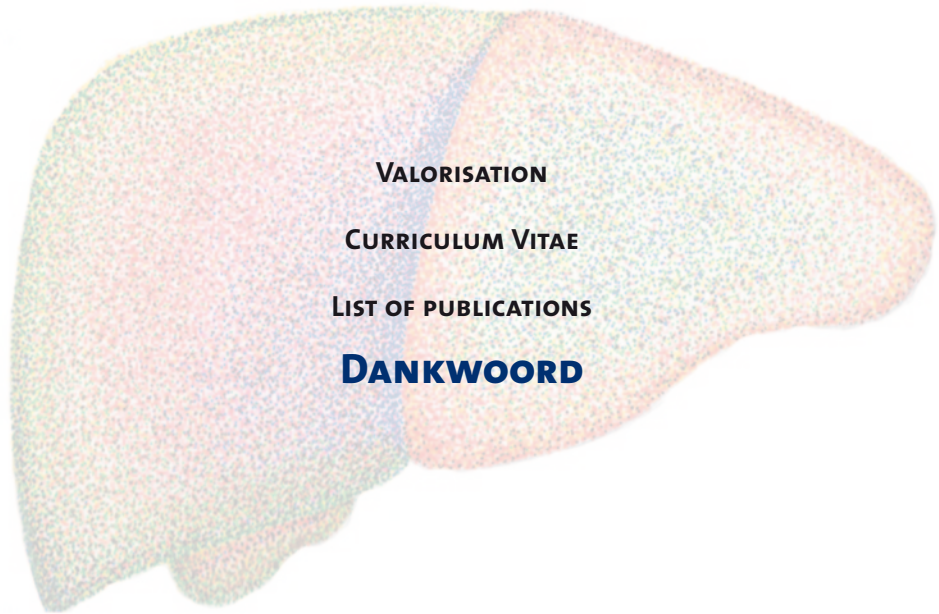
Van den Hof, W.F.P.M., Ruiz-Aracama, A., Van Summeren, A., Jennen, D.G.J., Gaj, S., Coonen, M.L.J., Brauers, K., Wodzig, W.K.W.H., van Delft, J.H.M., Kleinjans, J.C.S. A systems biology approach to unravel mechanisms of Cyclosporine A induced hepatotoxicity. Poster presentation at the 51st SOT Annual Meeting, March 2012, San Fransisco, USA.

Van den Hof, W.F.P.M., van Delft, J.H.M., Wodzig, W.K.W.H., Kleinjans, J.C.S. A metabolomic approach to predictive liver toxicology. Poster presentation at the Annual Meeting of the Dutch Society of Toxicology, May 2011, Zeist, the Netherlands.

Van den Hof, W.F.P.M., van Delft, J.H.M., Wodzig, W.K.W.H., Kleinjans, J.C.S. A metabolomic approach to predictive liver toxicology. Poster presentation at the 6th Metabolomics Society Conference, June 2010, Amsterdam, the Netherlands.

Achievements

Travel grant awarded by the Netherlands Society of Toxicology for attending the 51st SOT Annual Meeting, March 2012, San Fransisco, USA.



Dankwoord

Hier zit ik dan, 12 uur op een doordeweekse avond, is het dan waar? Is dit de laatste keer dat ik ’s nachts aan mijn thesis zal schrijven? Het is een eeuwenoud gezegde, maar het klopt, de laatste loodjes wegen het zwaarst. Maar nu ik begin aan mijn laatste hoofdstuk, zie ik waar ik het allemaal voor gedaan heb en ligt er gewoon een mooi boekje voor me. Natuurlijk is niet al het werk dat beschreven staat in dit boekje door mij alleen gedaan en het lijkt me dan ook vanzelfsprekend dat ik een heleboel mensen ga bedanken.

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Nadat alle experimenten waren uitgevoerd, moest de data uiteraard geanalyseerd worden. Dit was me nooit gelukt zonder de hulp van Maarten, Daniel, Danyel, Stan en Charly. Bedankt voor jullie scripts, excel hulp en advies voor alles wat met de data-analyse te maken heeft. Maarten, jou wil ik in het bijzonder bedanken aangezien jij de meeste analyses voor mij gedaan hebt. De publicatie over de classificatie van toxische stoffen was er zonder jouw hulp niet gekomen.

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